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Osteopontin Action on Adipose Tissue Macrophages and Adipocytes

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Verfasserin / Verfasser:	Karina Gollinger
Matrikel-Nummer:	0204341
Studienrichtung (lt. Studienblatt):	Molekulare Biologie
Betreuerin / Betreuer:	Johannes Nimpf, Thomas Stulnig
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Zusammenfassung

Mit der Adipositas geht eine chronische Entzündung des Fettgewebes einher, welche durch Infiltration mit Makrophagen und Sekretion inflammatorischer Zytokine entscheidend an der Entstehung der Insulinresistenz und damit des Typ 2 Diabetes mellitus beteiligt ist. Osteopontin (OPN) ist ein inflammatorisches Zytokin, welches hauptsächlich von Makrophagen produziert und im humanen sowie murinen Fettgewebe bei Adipositas besonders stark exprimiert wird. Experimente in Mäusen deuteten auf eine wichtige Rolle von OPN bei der Entstehung der Fettgewebsentzündung und der Insulinresistenz hin, die zugrunde liegenden Mechanismen sind jedoch noch nicht näher bekannt. Dementsprechend war das Ziel dieser Arbeit, die Zielzellen der OPN-Wirkung im Fettgewebe zu identifizieren und die damit verbundenen molekularen Vorgänge näher zu untersuchen, um dadurch Rückschlüsse über die Entstehung der Fettgewebsentzündung und Insulinresistenz zu erhalten. Zu diesem Zweck untersuchten wir die Wirkung von OPN auf humane Modellmakrophagen sowie isolierte humane Fettgewebsmakrophagen (ATMs), und humane und murine Zellkulturmodelle von Adipocyten.

Stimulation mit OPN führte in humanen Makrophagen zu gesteigerter Produktion von pro- und anti-inflammatorischen Zytokinen (TNFα, Mcp-1 bzw. IL-10). Diese Zytokine wurden auch von humanen ATMs produziert, wobei die Stimulierbarkeit durch OPN in Abhängigkeit von der entsprechenden Aufreinigungsmethode schwankte. Behandlung mit OPN aktivierte außerdem die MAP Kinasen ERK und p38 und die Serin/Threonin Proteinkinase PKB/Akt, welche die Expression inflammatorischer Gene steuern. Außerdem aktivierte OPN in geringerem Ausmaß den NFκB Signalweg und zwar unabhängig vom LPS/TLR-4/IRAK Signalweg. Im Gegensatz dazu hatte OPN praktisch keine Wirkung auf die Insulinsensitivität und Differenzierungskapazität der Adipocyten. Unsere Daten weisen darauf hin, dass die Wirkung von OPN bei der Adipositas-assoziierten Insulinresistenz primär über Anlockung und Aktivierung von Makrophagen und der damit verbundenen Fettgewebsentzündung, und nicht über direkte Interaktion mit Adipocyten erfolgt.

Abstract

Obesity gives rise to a state of chronic low-grade inflammation that contributes to insulin resistance and type 2 diabetes and is characterized by the infiltration of adipose tissue macrophages (ATMs) into adipose tissue (AT) along with production of inflammatory cytokines. Osteopontin (OPN) is an inflammatory cytokine that is mainly produced by activated macrophages and whose expression in human and murine AT is markedly upregulated upon obesity. Previous animal experiments strongly suggested a critical role of OPN in the development of insulin resistance and AT inflammation but the underlying molecular processes are unknown so far. In this thesis, we sought to identify the target cells of OPN action in the inflamed AT and, as a consequence, alterations induced in the target cells that may lead to insulin resistance. We investigated the effect of OPN on adipose tissue macrophages and adipocytes by using human model macrophages as well as isolated ATMs, and human and murine adipocyte model systems.

In both models of human macrophages, OPN induced secretion of inflammatory cytokines (TNFα, Mcp-1, IL-10), whereas human ATMs were also found to produce these cytokines, but responsiveness to OPN varied according to the isolation method used. In model macrophages, OPN treatment clearly induced activation of the MAP Kinase ERK, p38, and the serine/threonine kinase PKB/Akt, that are all associated with expression of inflammatory genes. The NFκB pathway was activated to a minor extent, notably independent from the LPS/TLR4/IRAK pathway. By contrast, OPN had no profound effect on insulin sensitivity or differentiation capacity of adipocytes.

Taken together, these findings suggest that the primary role of OPN in obesity-associated insulin resistance is the attraction and activation of macrophages with resulting AT inflammation, rather than direct interference with adipocyte function.

1 Introduction

1.1 Obesity

1.1.1 Physiological regulation of energy balance

The mechanisms involved in body weight regulation are versatile and include genetic, physiological and behavioural factors. The maintenance of an adequate body weight is a major determinant of the survival of higher organisms and has been particularly important during evolution in periods of starvation. To ensure stability of body weight over long periods of time, a complex regulatory system is necessary that adjusts energy intake with energy expenditure (1).

In healthy individuals, stable body weight is maintained by regulation of food intake and metabolic rate. Ingestion of food leads to a suppression of hunger and termination of food intake. This process is called satiation and is followed by a period that is characterized by the absence of hunger until the resurgence of the sensation of hunger (1, 2). Body size and composition also play a role in the control of energy balance: With undernutrition/weight loss, appetite increases and energy expenditure falls; with overfeeding/weight gain, appetite falls and energy expenditure increases (1, 3).

Such adaptive feeding and metabolic responses involve brain pathways that sense changes in body fuel stores and exert powerful effects on energy balance[1] (1, 4). The hypothalamus is the main regulatory organ for hunger and satiety in humans, integrating signals from the brain, the peripheral circulation and the gastrointestinal tract (5).

The ease with which many individuals gain weight seems inconsistent with the concept of a robust regulatory system that controls body weight (4). High prevalence rates of obesity worldwide suggest that our regulatory systems defend against weight loss more effectively than against weight gain (4, 6, 7): the need to survive in environments with limited access to food is a much higher concern than to handle an overabundant food supply, which was unfrequent in the past and an uncommon threat to survival (4, 6, 8, 9). Furthermore, it is evident that, in our modern civilizations, food intake is not necessarily the result of hunger. Numerous situations may lead to food or fluid

[1] energy balance = the net difference between energy intake and expenditure

consumption as a result of social activities. Due to this massive overfeeding in the absence of hunger, many people override their physiological mechanisms of weight regulation and become obese (1).

1.1.2 Epidemiology

The worldwide prevalence of overweight and obesity has increased drastically during the last decades, and is now representing a major public health issue: Excessive weight is associated with an array of serious medical conditions, including insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, cardiovascular disease, and some cancers, to name just a few examples (10).

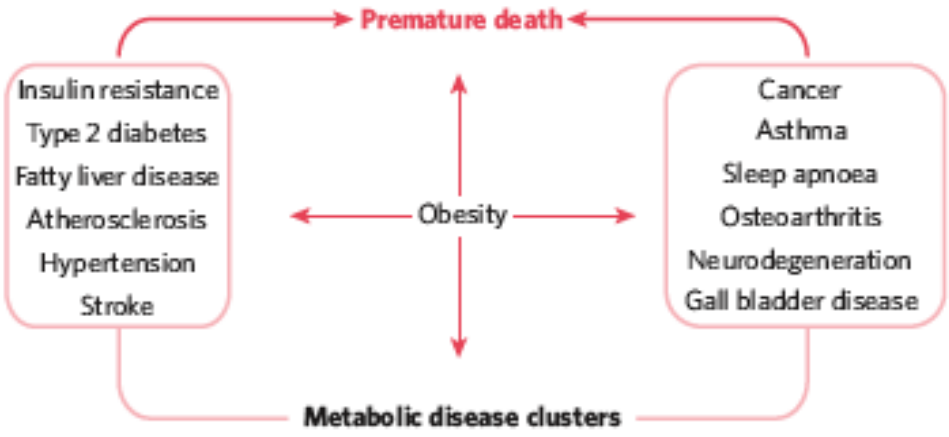

Figure 1. Clustering of metabolic diseases (10)

This cluster of pathologies has also started to emerge in children, which can be related to the similarly marked increase in obesity at young ages (10, 11).

Today, the World Health Organisation estimates that more than 1 billion adults worldwide are overweight, defined as having a BMI[2] of 25 to 29.9, or obese, defined by a BMI $\geq$ 30 (10, 12, 13). The rationale behind these definitions is based on epidemiological data that show increased mortality in subjects with BMIs $\geq$ 25 kg/m^2. This increase in mortality, however, tends to be modest until a BMI of 30 kg/m^2 is reached. Beyond this, mortality rates from all causes, and especially those from cardiovascular disease are increased by 50-100 % above that of lean subjects (12). If

[2] The BMI is calculated as follows: BMI = weight (kg)/ height squared (m^2)

prevalence rates continue to rise, obesity is predicted to surpass smoking as the leading risk-factor for mortality[3] within the next few years (14, 15). In some western countries as the United States, already more than 20% of the population can be classified as obese (16), demonstrating the enormous impact of obesity on public health issues.

These data are not surprising, though, considering the lifestyle of the modern western civilization: Industrialization and its economic consequences have led to an increasingly urbanized and sedentary workforce, coupled with excessive caloric consumption due to easy access to food (14). Today, obesity along with its consequences is considered to be the epidemic of the 21st century.

1.2 White adipose tissue

1.2.1 Anatomical features

Virtually all animal species have found a way to store excess energy in the form of fat for future needs. In most species, including humans, fat storage occurs predominantly in a mesodermal tissue, namely white adipose tissue (WAT), one of the two types of AT[4] (17).

AT is a complex, essential, and highly active metabolic and endocrine organ. In addition to adipocytes, it contains connective tissue matrix, nerve tissue, stromavascular cells, and immune cells (18, 19). White fat cells/adipocytes contain one large fat vacuole and are therefore also referred to as unilocular fat cells.

When an overabundance of energy is taken up, overweight or obesity develops, characterized by an expansion of AT. This enlargement of adipose mass is accomplished by an increase in adipocyte size (hypertrophy), and increase in fat cell number (hyperplasia) (19).

[3] in the United States

[4] The other one is brown adipose tissue (BAT) whose primary purpose is to generate body heat.

1.2.2 Adipogenesis

Adipocytes originate from mesenchymal/mesodermal stem cells by a sequential pathway of differentiation, i.e. adipogenesis. When subjected to appropriate developmental triggers, mesenchymal stem cells become committed to the adipocyte lineage. This differentiation presumably involves a common preadipocyte or adipoblast, which has the capacity to differentiate into white or brown preadipocytes (17).

The process of terminal adipocyte differentiation during which preadipocytes mature into adipocytes has been extensively studied in murine 3T3-L1 and 3T3-F442A cell lines (17, 20). The signal for differentiation of new adipocytes is related to nutritional state. Important stimuli for differentiation include insulin and fatty acids. Fatty acids promote adipogenesis through members of the peroxisome proliferator-activated receptors (PPAR, particularly PPARγ), a family of nuclear receptors, i.e. ligand-activated transcription factors. Natural ligands for PPAR are probably fatty acid derivatives (21, 22). In addition, differentiation is regulated by a pathway involving the sterol regulatory element binding protein-1c (SREBP-1c, also known as adipocyte determination and differentiation factor-1, ADD-1), a pathway that in adipocytes is regulated by insulin (21, 23, 24).

1.2.3 Fat distribution

In humans, WAT is dispersed throughout the body in discrete depots. A major distinction can be drawn between visceral fat, which surrounds internal organs (intestines and other intra-abdominal organs as well as perirenal areas), and subcutaneous fat which is found underneath the skin mainly in the buttocks, thighs and abdomen. Other fat depots that are of metabolic or cardiovascular relevance are intramuscular and epicardial fat (17).

Among these various AT depots, there is considerable heterogeneity, particularly with respects to metabolic risk (17, 18). Increased subcutaneous fat in the thighs and hips (peripheral or pear-shaped obesity) exerts little or no risk of metabolic disease, whereas increased intra-abdominal/visceral fat (central or apple-shaped obesity) promotes a high risk (17, 25). This observed discrepancy in disease risk may be due to several reasons: First, the anatomic location of the AT depot is affecting endocrine function and disease risk. Endocrine hormones derived from subcutaneous AT are secreted into the systemic

circulation, whereas those derived from visceral AT are secreted into the portal system and have direct access to the liver thereby affecting hepatic metabolism (18). In addition, metabolic activity is considered to vary between different AT depots: intra-abdominal adipocytes have the highest metabolic activity[5] followed by upper-body subcutaneous adipocytes, and lowest response in lower-body adipocytes (21, 26). Intra-abdominal adipocytes have the highest rates of lipolysis and lipolysis is least susceptible to suppression by insulin here. In contrast, lower-body fat depots are more efficient at removing fat from the circulation and due to a lower rate of lipolysis they are relatively resistant to weight loss (21).

Furthermore, adipokine expression and receptor expression patterns vary among different AT depots: For instance, expression and secretion of interleukin (IL)-6 and plasminogen activator inhibitor (PAI)-1 are relatively greater in visceral AT, whereas those of leptin and adiponectin is lower than in subcutaneous AT. Up to now, the precise mechanisms accounting for these differences are not fully elucidated, but this functional heterogeneity suggests that AT is probably rather a group of similar endocrine organs than one single endocrine organ (18).

1.2.4 Physiology

Until the late 1980s, AT was considered as a passive organ with the only function to be the storage of excess energy as triglycerides, and its degradation and release of these lipids in the form of fatty acids according to need (27).

Today, AT is known to exert, besides its role as energy store, important endocrine and paracrine function: AT is a major site for metabolism of steroid hormones and is secreting a variety of proteins that may either act locally or systemically to influence metabolism, endocrine, or immune function. In addition, AT expresses numerous receptors enabling it to respond to incoming signals, including those from the central nervous system (CNS). Hence, AT is involved in coordinating a variety of biological processes including energy metabolism, neuroendocrine function and immune function (18).

[5] i.e. here: lipolysis

1.2.5 Adipokines

AT is by now well recognized as an endocrine organ which secretes a number of signaling peptides with diverse biological functions, collectively called adipokines (28-31). They have important autocrine/paracrine roles in regulating adipocyte differentiation, metabolism, and local inflammatory responses (28, 30, 32-35). Adipokines also have important roles in the regulation of systemic lipid and glucose metabolism (28, 36-38) and the secretion of many adipokines is related to the degree of adiposity (28, 39-43).

1.2.5.1 Classical adipokines – Leptin and adiponectin

Leptin, a polypeptide with structural homology to cytokines, is the prototype for all adipose tissue-derived endocrine hormones (18).

Adipocytes secrete leptin in direct proportion to adipose tissue mass and nutritional status (18). Accordingly, its primary role – besides other endocrine functions - is to serve as a metabolic signal of energy sufficiency, primarily via hypothalamic pathways (18, 44). With caloric restriction and weight loss, leptin levels rapidly decline, leading to increased appetite and decreased energy expenditure, whereas massive obesity is often associated with elevated circulating leptin and an inability to respond to leptin (leptin resistance) (18, 45).

Adiponectin (AdipoQ) is specifically produced by adipocytes and circulates at high levels in the bloodstream (18, 46-50). In contrast to leptin, plasma adiponectin levels have been found to be decreased in states of obesity and insulin resistance and several studies have suggested an antidiabetic, antiinflammatory and antiatherogenic effect (18, 50, 51).

1.2.5.2 Inflammatory adipokines

1.2.5.2.1 Tumor necrosis factor (TNF)α

TNFα is an inflammatory cytokine that is secreted by the AT, mainly macrophages, but also adipocytes and other stromavascular cells (52). Its expression is increased in obese individuals and has been implicated in the pathogenesis of obesity and insulin resistance (53-56).

1.2.5.2.2 IL-6

IL-6 is another inflammatory cytokine that is expressed by the AT (57). In contrast to

TNFα, IL-6 circulates at high levels in the bloodstream, and both expression and circulating levels are positively correlated with obesity and insulin resistance (18, 56).

1.2.5.2.3 Monocyte chemoattractant protein (Mcp)–1

Mcp-1, a chemokine that recruits monocytes to sites of inflammation, is expressed and secreted by the AT. AT expression of Mcp-1 and circulating Mcp-1 levels are increased in rodent obesity, suggesting that Mcp-1-mediated macrophage infiltration of AT (see 1.4.2.3) may contribute to insulin resistance (18, 34, 58-61).

1.2.5.2.4 IL-10

IL-10 is an anti-inflammatory cytokine produced by various cell types, such as activated monocytes, macrophages, lymphocytes, and keratinocytes. Obesity is associated with elevated circulating levels of IL-10 and the expression of IL-10 is increased in obese human and rodent AT (62, 63). As an anti-inflammatory cytokine, IL-10 has been shown to have a protective role on the formation and stability of atherosclerotic lesions in rodents (62, 64), while human subjects with low IL-10 serum levels have an increased risk of developing type 2 diabetes mellitus (62, 65).

1.3 Insulin and insulin resistance

1.3.1 Functions of insulin

Insulin is the most potent anabolic hormone known and is essential for tissue development, growth, and maintenance of whole-body glucose homeostasis. It is secreted by the pancreatic β cells in response to increased circulating levels of glucose and amino acids after a meal (66). Insulin increases glucose uptake in muscle and fat and inhibits hepatic glucose production, thereby serving as the primary regulator of blood glucose, which is maintained at a concentration of 4 – 7 mM in healthy individuals. Insulin also promotes the storage of substrates in fat, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breakdown (67).

1.3.2 Insulin signaling

1.3.2.1 Insulin action

Insulin increases glucose uptake in cells by stimulating the translocation of the glucose transporter GLUT4 from intracellular sites to the plasma membrane (67). Insulin action is initiated through the binding to and activation of its cell-surface receptor (Figure 2). The insulin receptor is a tyrosine kinase that undergoes tyrosine autophosphorylation upon activation and thereafter phosphorylates a number of cellular proteins on tyrosine (66-68), including members of the insulin-receptor substrate (IRS) family (66, 69). The phosphorylated tyrosines in these substrates act as docking sites for proteins that contain Src-homology-2 (SH2) domains, such as the regulatory subunit of the type 1A phosphatidylinositol 3-kinase (PI(3)K) (67), the activation of which may transmit multiple signals. PI(3)K catalyzes for instance the phosphorylation of phosphoinositides to phosphatidylinositol-3-phosphates, especially PtdIns(3,4,5)P_3 which bind to the pleckstrin homology (PH) domains of a variety of signaling molecules (67, 70). Phosphatidylinositol-3-phosphates induce, among others, the activation of the serine/threonine kinase Akt, which plays an important role in the transmission of the insulin signal (67, 71-73). This and other pathways (74-77) act in a concerted fashion to coordinate the regulation of GLUT4 vesicle trafficking, protein synthesis, enzyme activation/inactivation, and gene expression, which results in the regulation of glucose, lipid and protein metabolism (67).

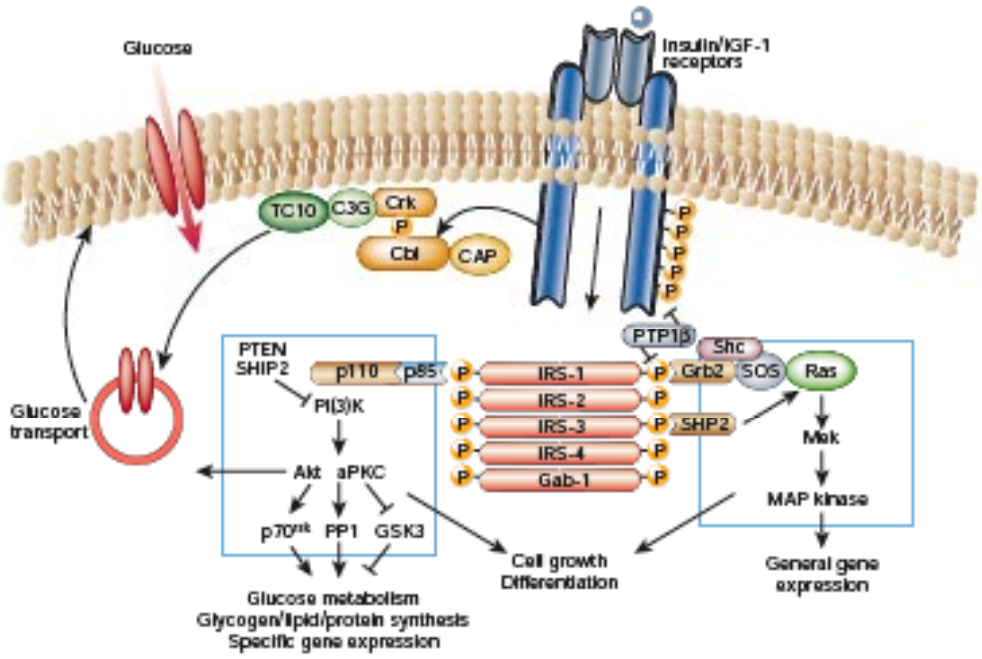

Figure 2. Signal transduction in insulin action (67)

1.3.2.2 Attenuation of insulin-receptor signaling

In addition to tyrosine autophosphorylation, both the insulin receptor and IRS proteins undergo serine phosphorylation, which impair insulin signaling (67, 78). Moreover, several protein tyrosine phosphatases (PTPases) catalyze dephosphorylation of the receptor and its substrates, thereby attenuating insulin action (66, 67).

1.3.3 Insulin resistance

1.3.3.1 Definition

Insulin sensitivity is defined as the ability of insulin to reduce circulating glucose concentrations. Accordingly, insulin resistance is a condition in which greater concentrations of insulin are necessary to produce an adequate reduction of plasma glucose concentrations and in severe insulin resistance, even very high insulin concentrations cannot evoke a maximal response of glycemia that is achieved under physiological concentrations.

1.3.3.2 Role of AT in insulin resistance

Obesity is an important factor for the development of insulin resistance. Adipose tissue controls insulin sensitivity by releasing nonesterified fatty acids (NEFAs), hormones – including adiponectin, leptin, and resistin – and proinflammatory cytokines (79-82). In obesity, the production of many of these substances is disregulated. Fatty acids are supposed to cause insulin resistance by inhibiting glycolysis, leading to increased intracellular glucose-6-posphate concentrations that result in decreased glucose uptake (79, 83). An alternative mechanism has been proposed in which intracellular fatty acid metabolites lead to phosphorylation of serine/threonine sites on IRS-1 and IRS-2 thereby reducing their ability to activate PI(3)K. As a consequence, glucose transport activity and other events downstream of insulin receptor signaling are diminished (79, 84).

In addition to fatty acids, increased release of TNFα, IL-6, Mcp-1, and other inflammatory products is also suggested to have a role in the development of insulin resistance (see 1.4.1).

1.4 AT inflammation

1.4.1 Link between obesity, inflammation and insulin resistance

During the past decade, it has become clear that obesity, insulin resistance and type 2 diabetes are closely associated with a state of chronic low-level inflammation. This is indicated by elevated plasma levels of C-reactive protein (CRP), IL-6 and PAI-1 in obese individuals (85-88).

The first molecular link between inflammation and obesity was provided in 1993 by Hotamisligil et al who showed that the inflammatory cytokine TNFα is overexpressed in the adipose tissue of obese rodents. Neutralization of TNFα led to a decrease in insulin resistance in these models of obesity (54). Later, TNFα overproduction of AT was also confirmed in obese humans, demonstrating the importance of this cytokine to obesity and insulin resistance (42, 89).

By now it is widely accepted that the adipose tissue acts as an endocrine organ by the secretion of cytokines and other bioactive substances, including Mcp-1, PAI-1, IL-6, resistin, and adiponectin, some of which directly decrease insulin sensitivity (34, 49, 57, 80, 90, 91). Moreover, it has been found several years ago that macrophages accumulate in obese white adipose tissue and that macrophages are the major producers of inflammatory mediators. This suggests a potentially important influence of AT macrophages in promoting insulin resistance (34, 60).

Nevertheless, it has remained unclear how inflammation in adipose tissue is initiated. It is assumed that the process is started in the adipocytes in which dietary excess and obesity cause lipid accumulation and cellular stress (80) (ROS (92, 93) and ER stress (94-96)) that lead to activation of the inflammatory signaling pathways of JNK (97, 98) and IKKβ/Nf-κB (99, 100) (Figure 3).

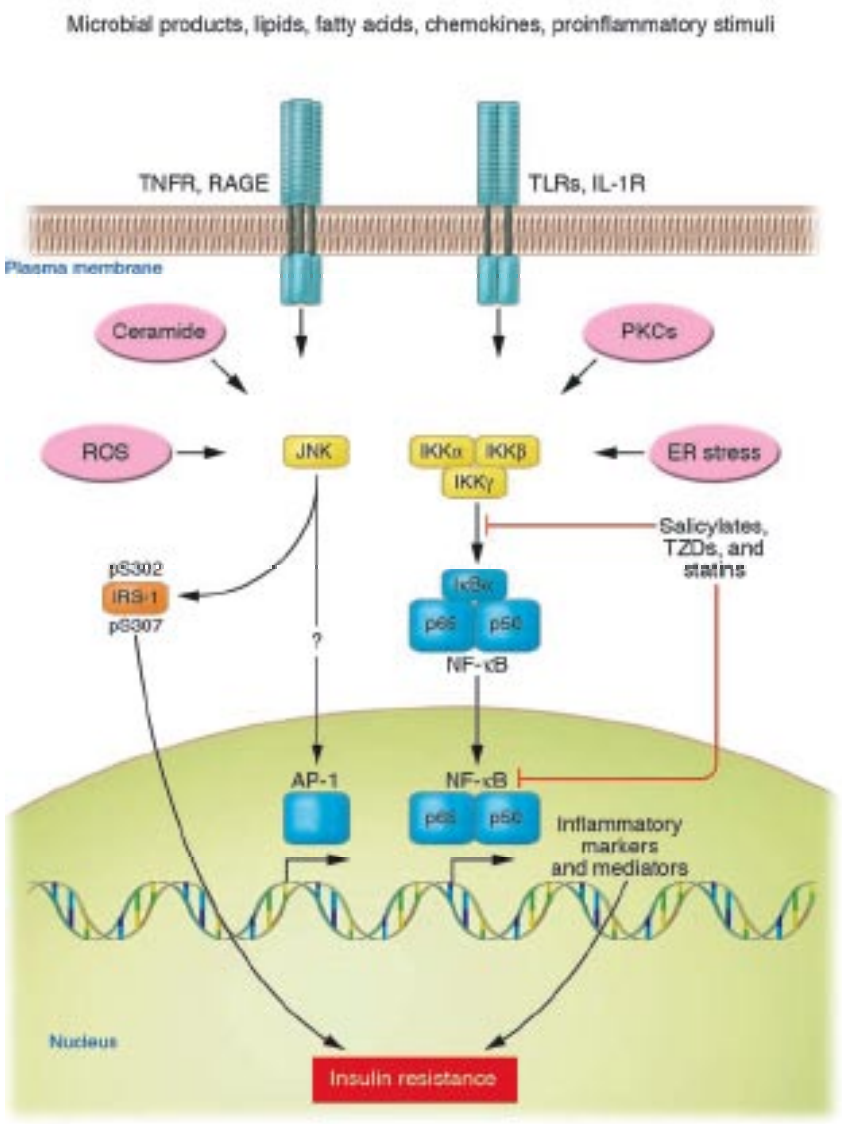

Figure 3. Potential cellular mechanisms for activating inflammatory signaling (80)

Obesity-induced IKKβ activation leads to NF-κB translocation and the increased expression of numerous inflammatory markers that may cause insulin resistance. JNK activation promotes phosphorylation of IRS-1 at serine residues that impairs downstream insulin signaling events, thereby promoting an insulin resistant state. Moreover, these inflammatory signaling pathways lead to increased adipocyte production of cytokines, including TNFα, IL-6 and chemokines such as Mcp-1 that attract monocytes into the adipose tissue where they differentiate into macrophages. The differentiated macrophages produce many of the same inflammatory cytokines and chemokines including but not limited to those listed above, to further promote and propagate the inflammatory response that ultimately results in insulin resistance (80).

1.4.2 Adipose tissue macrophages (ATMs)

1.4.2.1 Macrophages

Macrophages are phagocytic cells of the innate immune system and originate from myeloid progenitor cells in the bone marrow. They are distributed widely in the body tissues, where they play a critical part in innate immunity. Macrophages mature from monocytes, which circulate in the blood and differentiate into macrophages upon migration into tissues. Macrophages are the primary mediators of the innate immune response and also participate in adaptive immunity. They recognize and phagocytose foreign organisms, as well as dead cells and cell debris, release antimicrobial peptides, secrete molecules that attract other immune cells to areas of infection, and present antigens to lymphocytes. Macrophages express different receptors that recognize bacterial molecules and mediate the adhesion to other immune cells, endothelial cells and matrix proteins, such as CD14, the mannose receptor (MR), the scavenger receptor, the glucan receptor, the adhesion molecules CD44 and CD31, and the integrin chains CD11a, CD11b, and CD11c (101, 102).

1.4.2.2 Macrophage activation

According to the classical view, macrophages are stimulated by interferon-(IFN)γ alone or in combination with a microbial stimulus. IFNγ is produced by $CD4^+$ Th1 cells, $CD8^+$ T-cells, and natural killer cells in order to activate macrophages in innate and adaptive immune responses. Activated macrophages produce inflammatory cytokines (IL-1, TNFα, IL-6), reactive oxygen and nitrogen intermediates to kill the pathogens and are capable of inducing Th1-polarized T-cell responses (102). These classical macrophages are named M1.

Less well defined is the alternative activation of macrophages by IL-4 and IL-13, particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogens (103). Macrophages activated that way are referred to as M2 and are characterized by an upregulated expression of anti-inflammatory cytokines IL-1 receptor agonist and IL-10 leading to downregulation of inflammatory processes (104-107). They promote Th2 responses and accordingly exert poor anti-bacterial killing activity, but are highly active in particle uptake, which is reflected by expression of the non-opsonic pathogen receptors MR (CD206) (108) and the β-glucan receptor (109). Importantly, M1 and M2 macrophages as used in this thesis are regarded as only two

extremes of a continuum of functional stages of macrophages (103).

1.4.2.3 ATM infiltration in obesity and insulin resistance

Inflammatory cytokines are highly expressed in obese AT and promote the development of insulin resistance. Several lines of evidence have demonstrated that the main source of inflammatory adipokine (1.4.1) production in AT are nonfat cells, such as macrophages, that infiltrate the adipose tissue directly proportional to measures of adiposity (60, 110).

It is supposed that macrophages are recruited by chemotactic signals, such as Mcp-1, produced by the expanding adipocytes (82). Morphological examination of obese AT has revealed that macrophages localize around necrotic adipocytes, where they form so-called crown-like structures that probably sequester adipocyte debris (111). ATMs are therefore supposed to have clearance purposes, yet they may also reflect an attempt to limit the expansion of the adipocytes (10).

In vitro studies have shown that macrophage-secreted factors affect insulin sensitivity in adipocytes by altering expression of the glucose transport protein 4 (GLUT4) and IRS-1 in 3T3-L1 adipocytes (112, 113) and inhibit adipogeneses of preadipocytes (114). These findings suggest that ATMs critically contribute to the development of insulin resistance but their exact role in this process has not been fully delineated yet.

1.4.2.4 The nature of ATMs

Most experimental data on AT inflammation stem from murine experiments and in general, F4/80 expressing cells in murine AT are referred to as ATMs. Moreover, murine ATMs have been shown to be CD14 negative and CD11c has been found particularly as a marker for obesity-induced inflammatory (M1) ATMs (115, 116). Human ATMs markedly differ from murine ATMs by expressing CD14 whereas CD11c is only poorly expressed (115, 117, 118).

In a recent study of our lab, human ATMs were found to resemble M2 macrophages phenotypically by the expression of characteristical M2 surface markers (MR, CD163, integrin $\alpha v\beta 5$), their endocytic activity and the production of anti-inflammatory cytokines (IL-10, IL-1 receptor antagonist). By contrast, ATMs were also capable of secreting large amounts of inflammatory cytokines that are supposed to interfere with adipocyte function but the exact mechanisms leading to obesity-related diseases are not fully elucidated yet (118).

1.5 Osteopontin (OPN)

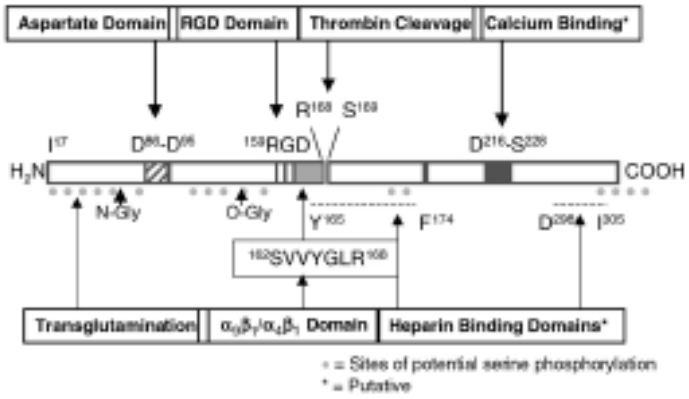

Figure 4. Some features of the OPN protein. Sites of O-glycosylation and phosphorylation are indicated; both vary (phosphorylation in particular) with the source of the protein. Numbering of the amino acids is based on the human protein (119).

1.5.1 Functions of Osteopontin

Osteopontin (Figure 4), also called secreted phosphoprotein 1 (SPP1) is a secreted matrix glycoprotein and inflammatory cytokine that has been implicated in a number of physiological and pathological events, including inflammatory processes (119). It is expressed by activated macrophages and T-cells, smooth muscle, endothelial, epithelial cells, and osteoclasts (120, 121). OPN was originally classified as Th1 cytokine and is involved in mineralization of bone and kidney, cell survival, tumor biology, and atherosclerosis (121, 122). It is secreted by macrophages at sites of inflammation and induces expression of inflammatory cytokines in peripheral blood mononuclear cells thereby playing a major role in cell-mediated immunity (123). It is known that OPN induces signaling pathways such as MEKK1/JNK1/AP-1 (124), IKK/IκB-α/NFκB (125, 126), and also FAK/PI(3)K/Akt (127). These data, however, have all been obtained from experiments in mice (tumor cells and pro-B-cells, respectively) and it was believed that some of these results may be due to LPS contamination of OPN preparations (128).

Its ability to interact with integrin surface receptors through an RGD sequence and with the CD44 receptor has established OPN as an important attachment and signaling molecule (119, 129, 130). It functions in cell migration, particularly of

monocytes/macrophages, and stimulates expression of matrix metalloproteases to induce matrix degradation and facilitate cell motility (120, 131, 132). Moreover, it is now well recognized that OPN plays a role in various inflammatory diseases, such as rheumatoid arthritis (133), atherosclerosis (134), and cardiac fibrosis (135).

1.5.2 Osteopontin in obesity and insulin resistance

Previous data from our group indicated considerable upregulation of the OPN gene *spp1* in the AT of obese mice and humans, and ATMs were found to be the main source of OPN production (131, 136). Further animal studies by us and others revealed improved insulin sensitivity of high-fat diet-induced OPN deficient mice and decreased AT inflammation without any effect on body mass or energy expenditure. Moreover, Nomiyama et al observed decreased ATM infiltration in these mice (129). These results strongly suggest a key role of OPN as a link of obesity and insulin resistance by promoting AT inflammation.

2 Aim of the study

Obesity gives rise to a state of chronic low-grade inflammation that contributes to insulin resistance and type 2 diabetes. This inflammation is characterized by an increased infiltration of macrophages into AT along with production of inflammatory cytokines that are thought to play a major role in the origin of inflammation.

Animal experiments in our laboratory showed that the OPN gene, *spp1*, is exceptionally upregulated in the AT of high-fat diet-induced obese mice and that *spp1* knockout mice show improved insulin sensitivity. These results and the fact that OPN is mainly produced in activated macrophages point towards a potential role of OPN in obesity-associated inflammation and insulin resistance.

As a result, it is the aim of this thesis to elucidate the mechanisms by which OPN contributes to AT inflammation and insulin resistance. Here we investigate if and how OPN interacts with cells of the AT, ATMs and adipocytes. To address these questions, we make use of human model macrophages and ATMs, murine wt and *spp1* knockout ATMs, human and murine adipocytes as well as murine ATM/adipocyte coculture systems.

3 Materials and Methods

3.1 Generation of human model macrophages

3.1.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Buffy coats from healthy donors (obtained from LKH Graz) were diluted 1:1 with Dulbecco's phosphate-buffered-saline (PBS; Cambrex). 15 ml Ficoll Paque Plus (Amersham Pharmacia) was placed in 50 ml tubes and 35 ml of diluted blood carefully placed on top. Tubes were centrifuged for 30 min (500 x g, RT, low brake) and the interphase containing mononuclear cells was collected, transferred into fresh 50 ml tubes, and washed twice with cold PBS at 500 x g, 7 min, 4 °C. Pellets were hemolysed in hemolysis buffer (0.15 M NH_3Cl, 0.1 M $KHCO_3$, 500 mM EDTA) for 7 min on ice and washed with PBS at 1,600 rpm, 7 min, 4 °C. Cells were resuspended and counted in 35 ml MACS buffer (0.5% BSA, 125 mM EDTA in PBS).

3.1.2 Separation of monocytes ($CD14^+$ cells) by magnetic cell sorting

Isolated PBMCs were washed twice in MACS buffer at 300 g, 10 min, 4 °C and resuspended in 800 µl MACS buffer and 200 µl CD14 microbeads (Miltenyi) per approx. 100 x 10^6 cells, mixed well and incubated on ice for 15 min. Samples were washed at 300 g, 10 min, 4 °C and resuspended in 500 µl MACS buffer per 100 x 10^6 cells. LS columns (Miltenyi) were placed on the MACS seperator (quadro MACS, Miltenyi) and equilibrated with 3 ml MACS buffer. Samples were applied onto the column and washed three times with 3 ml MACS buffer. After removing the column from the seperator, the fraction containing the magnetically labeled $CD14^+$ cells was immediately flushed out in 5 ml MACS buffer by firmly applying the plunger supplied with the column.

3.1.3 Macrophage differentiation

In order to obtain model macrophages, M1 (IFNγ) and M2 (IL-4) macrophages were differentiated from $CD14^+$ sorted PBMCs (monocytes). 2.5 x 10^6 cells per well of a 6-well plate were cultivated in 4 ml RPMI 1640 with 50 µg/ml streptomycin, 50 U/ml penicillin (all invitrogen) and 10% fetal calf serum (FCS; Hyclone), supplemented with 100 ng/ml IFNγ (Strathmann) or 10 ng/ml IL-4 (R&D Systems) to induce differentiation into M1 and M2 macrophages, respectively. Cells were kept in this medium for 5 days and thereafter used for experiments.

3.2 Isolation of human ATMs

3.2.1 AT fractionation

Subcutaneous AT (abdomen) was obtained from patients undergoing reductional plastic surgery. Omental AT was obtained from organ donors. This study was approved by the ethics committee of the Medical University Vienna and the General Hospital Vienna.

Samples were repeatedly rinsed in PBS to remove any blood. Blood vessels, fibrous material and connective tissue were carefully dissected and AT was cut into pieces of approx. 5 x 5 mm. AT was transferred into 50 ml tubes (10 g per tube) with 25 ml RPMI 1640 including Liberase Blendzyme 3 (Roche) at 35 µg/ml and DNase I (Sigma) at 50 U/ml and was incubated for 1 hour at 37 °C and 80 rpm. The digested tissue was passed through a 70 µm nylon mesh filter (Becton Dickinson, BD) and centrifuged at 1,000 x g, 10 min, 4 °C. Floating cells (adipocytes) and supernatants were removed. The pellet comprised the stromal vascular cell (SVC) fraction[6]. The SVC pellets were resuspended in 2 ml hemolysis buffer per tube and incubated on ice for 5 minutes. Subsequently, 45 ml cold PBS was added, the suspension passed again through a 70 µm filter and centrifuged at 500 x g, 7 min, 4 °C.

[6] The SV fraction is a mixed population of stromal cells comprising ~ 40 – 60% macrophages, preadipocytes, fibroblasts, and endothelial cells.

3.2.2 ATM isolation by MACS

Hemolysed pellets were resuspended in 1 ml Beriglobin (unspecific human antibodies, ZLB Behring, Vienna, Austria) per approximately 10 x 10^6 cells and cells were counted with a Bürker-Türk counting chamber. For flow cytometry-analysis, 100,000 – 200,000 cells per sample were pipetted on ice into a 1.4 ml u-tube (Micronic). After centrifugation at 500 x g, 3 min, 4 °C the pellet was resuspended in 80 µl MACS buffer and 20 µl CD14 MicroBeads (Miltenyi) per 10 x 10^6 cells and incubated for 15 minutes on ice. Samples were washed twice with 1 ml MACS buffer and resuspended in 500 µl (up to 10^8 cells) MACS buffer. Magnetic cell sorting was performed as described in 3.1.2 with the modification that MS columns were used which were equilibrated and washed with 500 µl MACS buffer. Cells were flushed out with 1 ml MACS buffer. To obtain higher purity the MACS-separation was repeated. Approximately 50,000 ATMs were used for flow cytometry-analysis to determine purity, which was 75 – 80 % $CD14^+$ cells.

3.2.3 ATM isolation by FACS

Hemolysed SVC pellets were resuspended in 50 µl MACS buffer and 25 µl of FITC-labeled CD14 and PE-labeled CD206 (MR) anti-human antibodies (both BD) per 10 x 10^6 cells. Samples were incubated on ice for 45 minutes and washed in PBS at 300 x g, 7 min, 4 °C. The Pellet was resuspended in 4 ml PBS, passed through a 70 µm filter and transferred into a FACS u-tube. Cells were sorted in FACSVantage or FACSAria (both BD) for $CD14^+MR^+$ and $CD14^+MR^-$ and taken up in 3.5 ml of culture medium. Sorted cells were centrifuged at 300 g, 5 min, 4 °C and resuspended in 400 µl culture medium. Approximately 20 µl of samples prior and post sorting were analyzed in FACSCalibur (BD) for purity of isolated populations. For isolation of RNA, sedimented cells were taken up in 0.5 ml TRIzol Reagent (invitrogen) and stored at –20 °C.

3.3 Quantitation of cytokine secretion

3.3.1 Cell stimulation

ATMs, M1s and M2s were stimulated with OPN and, for comparison, other cytokines and cell-activators to quantify their cytokine secretion. IFNγ and TNFα were purchased from R&D systems, LPS and OPN from Sigma. 100,000 cells per well of a 96-well plate were incubated for 2 days (M1 and M2) or 3 days (ATMs) at 37 °C in 200 µl culture medium supplemented with cytokine concentrations indicated in the results section. Subsequently, cell free supernatants were collected and stored at -20 °C for analysis of cytokine and chemokine concentrations.

3.3.2 Multiplexed microsphere-based flow cytometry

Cell free supernatants of ATMs, M1 and M2 were assayed with Human Fluorokine MultiAnalyte Profiling Kits (R&D Systems) for IL-10, TNFα and Mcp-1 according to the manufacturer's instruction. Briefly, 50 µl of the diluted microparticle mixture (6 x 10^4 beads/ml in 1% BSA) were added to each well of the microplate. 50 µl of standard or sample per well were added. The assay was incubated on a horizontal orbital microplate shaker at 500 rpm, 3 h, RT. Subsequently, samples were washed 3 times with 100 µl PBS and 50 µl of diluted biotin-conjugated antibody cocktail was added to each well. Dilutions used were 1:250 for IL-10, 1:400 for TNFα and 1:100 for Mcp-1 in 1% BSA. The plate was again incubated at 500 rpm, 1 h, RT and washed as described above. 50 µl of diluted streptavidin-PE [4 µl/ml in 1% BSA] was added to each well and the plate again incubated at 500 rpm, 30 min, RT and washed two times with PBS. The microparticles were resuspended by adding 90 µl PBS to each well and the assay was read by using a Luminex 100 IS^{TM} analyzer.

3.4 Western Blot

3.4.1 Stimulation and lysis of macrophages

Differentiated M1 and M2 macrophages as described in 3.1.3 were harvested by using a scraper and centrifuged at 500 g, 7 min, 4 °C. Pellets were resuspended in culture medium to approximately 10 x 10^6 cells/ml and counted by using a Bürker-Türk counting chamber. Aliquots of 150 µl containing at least 0.5 x 10^6 cells were stimulated with 1 µg/ml OPN, 100 ng/ml LPS, or left untreated for indicated time periods on a 37 °C waterbath. Cells were pelleted for 20 s and lysed in 50 µl lysis buffer for 30 minutes on ice (20 mM Tris/HCl pH 7.4, 140 mM NaCl, 1 % NP-40, 10 µg/ml aprotinin, 5 mM lodoacetamide, 10 µg/ml leupeptin, 1 µM pepstatin, 0.4 mM Pefabloc, 1 mM sodium orthovanadate, 10 mM NaF, 2.5 mM EDTA, 5 mM sodium pyrophosphate, 25 mM B-glycerolphosphate). Samples were spun down for 30 s and supernatants mixed with 15 µl 4 x sample buffer (240 mM Tris/HCl pH 6.8, 400 mM DTT, 8 % SDS, 44 % glycerol, 0.02 % Bromphenol-blue), heated at 99 °C for 3 minutes and stored at -20 °C.

3.4.2 SDS-PAGE

10 % acrylamidgels were made by injecting a 10% running gel solution (0.375 M Tris/HCl pH 8.8, 10% Acrylamid/Bis Solution (BioRad), 0.005 % APS, 0.004 % TEMED) into a BioTrade gel pouring chamber and overlaying it with 2-Butanol. Polymerized running gels were washed with water, dried by using a filter paper and overlaid by a 4 % stacking gel (0.125 M Tris/HCl pH 6.8, 4 % Acrylamid/Bis Solution, 0.001% APS, 0.0008 % TEMED). 14-well combs were inserted into the stacking gel and removed when gels were polymerized. Gels were placed into a running chamber and covered with running buffer (25 mM Tris, 192 mM Glycine, 0.1 % SDS). Slots were loaded with 7 µl prestained Molecular weight marker (BioRad Broad Range, 6 – 203 kDa) and 14 µl sample, respectively and gels were run at 130 V for 1.5 h.

3.4.3 Blot

Proteins were blotted onto a nitrocellulose membrane (Amersham Hybond ECL) by a semi-dry blotting apparatus (C.B.S. apparatus, BioTrade) for approximately 1 hour at maximally 23 V, limited to 0.1 A/gel. Ponceau Red staining was performed in order to verify successful operations and uniform protein concentrations in samples.
Blots were analyzed by use of a BM Chemiluminescence Blotting Substrate (POD) Kit (Roche). Briefly, membranes were blocked for 1 h in a 1:5 dilution of blocking reagent, washed twice in TBS-T (0.05 M Tris, 0.14 M NaCl, 0.05 % Tween, pH 7.5) and incubated with primary antibodies O/N at 4 °C. The following day, blots were washed as described above, blocked for another 10 min and incubated with secondary HRP-conjugated antibodies for 45 minutes RT protected from light. Thereafter, blots were washed three times with TBS-T and covered with substrate (1:100 dilution of Luminescence starting solution B in substrate solution A) for 2 minutes. Chemiluminescence was measured by a Lumi Imager (Roche; exposure time 10 minutes). Following antibodies and dilutions were used: IRAK-1 (Santa Cruz, rabbit anti-human, 1:100), IκB-α (Calbiochem, rabbit anti-human, 1:500), p-ERK (Santa Cruz, mouse anti-human, 1:500), ERK-2 (Santa Cruz, mouse anti-human, 1:2,000) p-Akt (Biovision, rabbit anti-human, 1:500), Akt (Biovision, rabbit anti-human, 1:500), p-p38 (Cell Signaling, rabbit anti-human, 1:500), p38 (Santa Cruz, mouse anti-human, 1:500), pJNK (New England Biolabs, rabbit anti-human, 1:500), goat anti-rabbit-HRP-conjugated secondary antibody (Accurate, 1:10,000), goat anti-mouse-HRP-conjugated secondary antibody (Biorad, 1:3,000).

3.5 Intracellular staining of MAP Kinase phosphorylation

Differentiated M1 macrophages were harvested and stimulated with OPN or LPS as described in 3.4.1. After incubation at 37 °C, the reaction was stopped with cold PBS. Samples were spun down for 20 s at 4 °C and supernatants removed. Cells were fixed and permeabilized using a Fix & Perm Cell Permeabilization Kit (An der Grub). Briefly, cells were fixed in 100 µl solution A for 15 min at RT and washed twice with PBS at 1,800 rpm, 3 min, RT. Subsequently, 50 µl permeabilization solution B and 1 µl primary antibody were added and samples incubated for 30 min at RT. Cells were washed twice with MACS buffer as described above and incubated in 20 µl of stained

secondary antibody dilution. After the incubation time of 30 min at RT, cells were washed with MACS buffer and PBS and resuspended in 200 µl PBS. Samples were analyzed in FACSCanto II (BD).
Following antibodies were used: rabbit anti-human p-JNK (Cell Signaling), secondary AlexaF488-conjugated goat-anti-rabbit IgG (invitrogen, 1:400 dilution). All obtained values were corrected for background fluorescence by staining with secondary antibody only.

3.6 Analysis of murine ATMs

3.6.1 Animals and animal care

To stimulate diet-induced obesity, male C57BL/6J wt or *OPN*$^{-/-}$ *(spp1)* mice (Charles River Laboratories, Sulzfeld, Germany) aged 7 weeks were placed on a high-fat diet (60 kcal percent fat, D12450B, Research Diets Inc., New Brunswick, NJ) for 20 weeks. Mice kept on a low-fat diet (10 kcal percent fat, D12492, Research Diets Inc.) served as lean controls. Animals had free access to food and water and were maintained on a 12-h light, 12-h dark cycle. After 20 weeks on the diet, mice were killed by cervical dislocation. The study protocols were approved by the local ethics committee for animal experiments, and followed the guidelines on accomodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

3.6.2 ATM isolation and FACS analysis

Epididymal fat pads from male C57BL/6J wt or *spp1* knockout mice fed a LFD or HFD were excised and minced in 5 x 5 mm pieces. 0.5 g tissue/ml was digested with 35 µg/ml Liberase Blendzyme 3 (Roche) and 50 U/ml DNase I (Sigma) in RPMI 1640 for 45 min at 37 °C, 80 rpm. Digested tissues were passed through 250 µm mesh filters. After centrifugation at 1,000 x g, 7 min, 4 °C, the stromal vascular pellets wer lyzed in

1 ml of hemolysis buffer and remaining cells passed through a 70 µm filter and centrifuged at 500 x g, 3 min, 4 °C. For flow cytometry analysis, pellets were resuspended in Beriglobin to block Fc-receptors and stained for 45 minutes on ice protected from light. Following antibodies were used: CD44-PE-Cy5 (BD), F4/80-FITC (AbD Serotec), CD11c-APC (BD), CD206-Alexa488 (AbD Serotec), CD51-PE (BD), all as a 1:30 dilution in Beriglobin. Cells were washed in PBS at 500 x g, 3 min, 4 °C and resuspended in 200 µl PBS. Samples were analyzed in FACSCalibur (BD).

3.7 Adipocyte cell culture

3.7.1 3T3-L1

3T3-L1 adipocytes were generated as described previously (137).
3T3-L1 fibroblasts were generously provided by Karsten Kristiansen, Denmark. Cells were cultured in DMEM (invitrogen) containing 4,500 mg/l glucose, 110 mg/l sodium pyruvate, 4 µg/ml pantothenate supplemented with 2 mM L-Glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin (all invitrogen), 4 µg/ml pantothenate and 8 µg/ml biotin (both Sigma). Standard proliferation medium contained 10% bovine calf serum (invitrogen) and cells were maintained at less than 30% confluence. Differentiation of 3T3-L1 cells into adipocytes was induced by exposing 2-3 d postconfluent (designated day 0) cells to DMEM containing 10% FBS (invitrogen) supplemented with 500 nM dexamethasone, 500 µM IBMX, and 1 µg/ml insulin from bovine pancreas (all Sigma). At Day 2, cells were fed DMEM containing 10% FBS and 1 µg/ml insulin; after another 2-4 days cells were kept in DMEM and 10% FBS. Adipocytes were used in experiments at Day 8 – 10 of differentiation.

3.7.2 Generation of human adipocytes

3.7.2.1 AT fractionation and preadipocyte isolation

Subcutaneous AT was obtained from patients undergoing reductive plastic surgery

(abdomen and 1 back). Basically, stromal vascular cells containing preadipocytes were isolated as described in 3.2.1 with some modifications: Minced AT was digested in a solution of 2 mg/ml Collagenase Type IV in Hanks' buffered salt solution (HBSS, both Sigma), 0.01 M HEPES supplemented with 50 µg/ml streptomycin, 50 U/ml penicillin (both invitrogen) and 25 µg/ml amphotericin B (Sigma). Tissue was digested as described and filtered through a 250 µm nylon mesh. The cell suspension was centrifuged at 200 x g, 10 min, 4 °C. Erythrocytes were hemolysed as above and SVCs were pelleted at 300 x g, 7 min, 4 °C. Cells were resuspended in DMEM/Ham's-F12 medium (Sigma, including 15 mM sodium carbonate and 15 mM HEPES) supplemented with 20% FCS (Hyclone), 50 µg/ml streptomycin, 50 U/ml penicillin and 25 µg/ml amphotericin B, referred to as proliferation medium.

3.7.2.2 Culture and differentiation of preadipocytes

Human preadipocytes were generated as described previously (138) with some modifications: Stromal vascular cells were seeded at a density of 100,000/cm^2 in 12-well plates (Corning Costar, CellBIND surface), each well representing ~ 4.5 cm^2. After 16 – 20 h routinely used for cell attachment, cells were carefully washed with PBS to remove nonadhering material, mainly white blood cells and cell debris, and fed with proliferation medium. Preadipocytes were grown to confluence for a maximum of 4 days, then washed twice with PBS to remove any serum that may interfere with differentiation. To induce differentiation of preadipocytes, cells were incubated in a chemically defined serum-free medium consisting of DMEM/Ham's F-12, 33 µM biotin, 17 µM pantothenate, 1 nM triiodothyronine, 100 nM dexamethasone (all Sigma), 500 nM human insulin (Roche), 1 µM rosiglitazone (generously provided by Johnson & Johnson), and, for the first 3 days, 250 µM IBMX (Sigma). To investigate a putative effect of OPN on preadipocyte differentiation, 500 nM OPN was added to the differentiation medium. Half of the medium was changed every 2 days and cells were used for experiments at day 9 – 11 of differentiation (Figure 5). 2 days prior to the experiment (glucose uptake), dexamethasone, insulin, and rosiglitazone were removed from the medium.

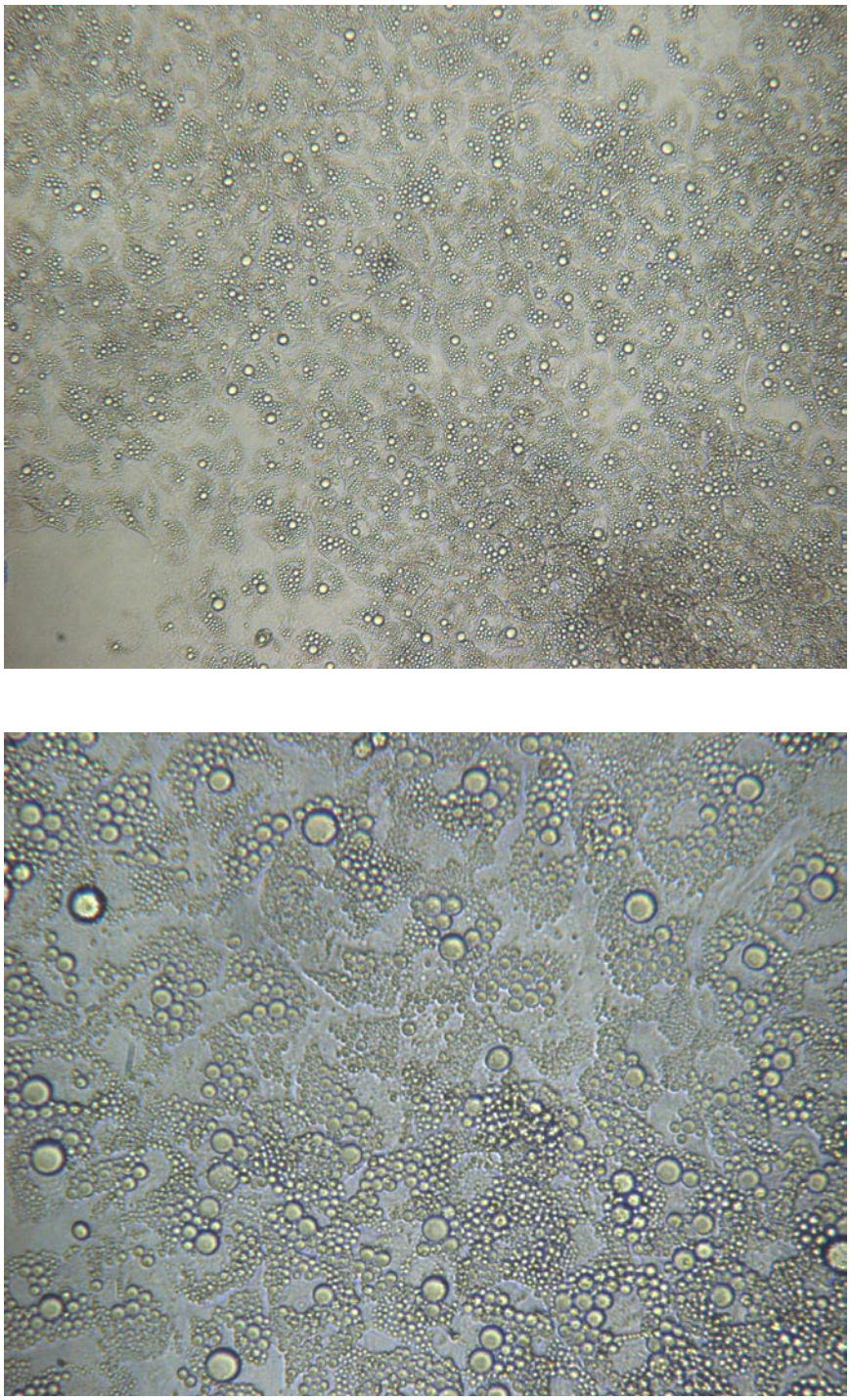

Figure 5. Morphology of human adipocytes differentiated for 14 days in culture.

3.7.3 Coculture of macrophages and adipocytes

Murine SVC from C57Bl/6J wt or *spp1* fed a HFD or LFD were isolated as described in 3.6.2. Cells were resuspended in growth medium at a concentration of approximately 100,000 cells/ml. Cells were allowed to attach on 12-well cell culture inserts (BD, pore

size 0.4 µm) at a concentration of 100,000 cells/well for 2 h at 37 °C. Nonadherent cells were washed away with PBS and inserts were added to differentiated 3T3-L1 adipocytes (6 – 8 days after induction of differentiation) for 48 h prior to the experiment.

3.7.4 2-Deoxyglucose uptake assay

3.7.4.1 3T3-L1

To investigate the effect of OPN and other substances on insulin sensitivity, differentiated 3T3-L1 adipocytes were pretreated with concentrations indicated in the results section 48 h prior to the assay. During the last 12-14 hours of the treatment (over night), cells were starved in serum-free DMEM 1,000 mg/l glucose supplemented with 0.5% BSA, 50 µg/ml streptomycin and 50 U/ml penicillin (starving medium) before insulin stimulation. In case of macrophage/adipocyte coculture, inserts were removed and adipocytes were starved in starving medium 3 h prior to insulin stimulation without OPN-treatment. After the starving period, cells were washed twice with Krebs-Ringer-Phosphate-HEPES (KRPH) buffer (136 mM NaCl, 5 mM Sodium phosphate buffer pH 7.4, 20 mM HEPES, 4.7 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$) and incubated with or without 1 µM insulin from bovine pancreas (Sigma) in 0.5 ml KRPH buffer for 30 min at RT. Subsequently, 1 µCi [^{3}H]2-deoxyglucose (Amersham Pharmacia) was added to all wells, i.e. 25 µl of a 20 µCi/ml stock solution and incubated for 10 min at RT. The [^{3}H]2-deoxyglucose stock solution was supplemented with 1 mM 2-deoxyglucose (Sigma) to provide glucose concentrations of 50 µM during the reaction. Glucose uptake was terminated by three washes with ice-cold PBS and the cells were lysed in 500 µl 0.2 N NaOH. 300 µl cell lysate were added to a scintillation vial containing 4 ml Ultima Gold High Flash Point LSC-cocktail (Perkin Elmer) and radioactivity was measured by a beta scintillation counter. To determine the total counts per minute, 2 µl of [^{3}H]2-deoxyglucose stock solution were mixed with 4 ml LSC-cocktail and radioactivity was counted.

Protein concentrations of the lysates were determined by a BCA Protein Assay Kit (Pierce) with BSA as the standard and data was expressed as pmol radioactive glucose taken up per 5 minutes and per mg protein (pmol/5 min/mg protein).

3.7.4.2 Human adipocytes

The glucose uptake assay was performed essentially as in 3.7.4.1 with the modification that adipocytes were not starved, and 1 µM human insulin was added directly to 0.5 ml medium and incubated for 15 min at 37 °C. Since glucose was already present in the medium, the [^{3}H]2-deoxyglucose stock solution was not supplemented with 2-deoxyglucose. Cells were incubated with 1 µCi [^{3}H]2-deoxyglucose for 20 min at 37 °C, washed and lysed as described above.

3.8 Gene expression analysis

3.8.1 RNA isolation

Human adipocytes at day 10 of differentiation were harvested in 1 ml Trizol, human ATMs as described in 3.2.3 were resuspended in 0.5 ml Trizol and both stored at -20 °C. RNA was isolated according to the manufacturer's protocol: 500 µl sample were transferred into Phase Lock Gel Heavy Tubes (Eppendorf Austria), mixed with 100 µl chloroform and 120 µl RNase-free water and incubated for 10 minutes at RT. Samples were centrifuged at 13,000 rpm, 15 min, 4 °C, and 380 µl of the RNA-containing aqueous phase was transferred into fresh tubes. 1 µl GlycoBlue (Ambion) was added to each sample and RNA was precipitated with 280 µl cold isopropanol for 30 min on ice. Samples were centrifuged at 13,000 rpm, 30 min, 4 °C. The RNA pellets were washed twice with 75% RNase-free ethanol at 10,000 rpm, 5 min, 4 °C, dried and dissolved in 22 µl RNase-free water.

3.8.2 cDNA synthesis

22 µl of isolated RNA (~ 1 µg) were mixed with 17 µl PCR mastermix containing First Strand Buffer, 12.5 mM DTT, 625 µM dNTPs, 187.5 ng/µl Random Hexamers and 50 U RNase Out Ribonuclease Inhibitor (all invitrogen). RNA secondary structure was denaturated for 15 minutes at 65 °C. Thereafter, 200 U of Super Script II Reverse Transcriptase (invitrogen) were added to the reaction and RNA was reverse transcribed into cDNA by 10 min of primer annealing at 25 °C and 50 minutes of reverse transcription at 42 °C. Finally, the reaction was heated at 70 °C for 15 minutes to inactivate the enzyme.

3.8.3 Quantitative Real-Time PCR (RT-qPCR)

RNA expression was quantified by RT-qPCR using an ABI Prism 7000 cycler (Applied Biosystems) and commercial Assay-on-demand kits for human TNFα, spp1, IL-10, CCL-2 (Mcp-1), SLC2A4 (GLUT-4), PPARγ, LIPE, and adiponectin (all Applied Biosystems) according to the manufacturer's protocol. Each PCR reaction contained, in a final volume of 25 µl, 0.625 µl of first-strand cDNA, 12.5 µl 2 x TaqMan Universal PCR Master Mix, 1.25 µl 20 x TaqMan Gene Expression Assay (both Applied Biosystems) and 10.625 µl H_2O. All reactions were performed using the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. PCR was carried out in duplicates in MicroAmp Reaction Optical 96-well plates (Applied Biosystems). Target gene mRNA expression was normalized to 18S rRNA and the relative amounts of all mRNAs were calculated using the comparative ΔC_T method.

3.9 Statistics

All values are given in mean ± SE. Significance of differences between two groups were assessed by the unpaired Student's *t* test. For analyses of more than two groups, One-way Anova and post-hoc Dunnett tests were used. $P < 0.05$ was considered as statistically significant.

4 Results

4.1 OPN-induced macrophage activation

4.1.1 OPN stimulates signal transduction in M1 macrophages

As described in 1.4.2.2, we used classical M1 as well as alternatively activated M2 macrophages as model ATMs. To investigate a putative effect of OPN on the activation of signaling pathways in macrophages, M1 were stimulated with OPN and, for comparison, LPS for 15', 30', and 60'. Data for M2 macrophages is omitted because no activation of signaling pathways was detected upon LPS stimulation. After 15 minutes of stimulation, IRAK-1 is degraded if activated by LPS, but not OPN, thereby indicating that OPN signals independently from the LPS/TLR4/IRAK pathway. In contrast, OPN induced phosphorylation of the MAP Kinases p38 and ERK-2, as well as Akt and, to a minor extent, degradation of IκB-α (Figure 2). Interestingly, OPN-induced activation of signaling pathways was delayed in comparison to LPS, but reached, and in case of pERK even exceeded the strength of LPS-induced events after 60 minutes of stimulation. Phosphorylation of JNK was strongly induced by LPS after 15 minutes of stimulation, but only weakly in OPN-stimulated cells (Figure 6).

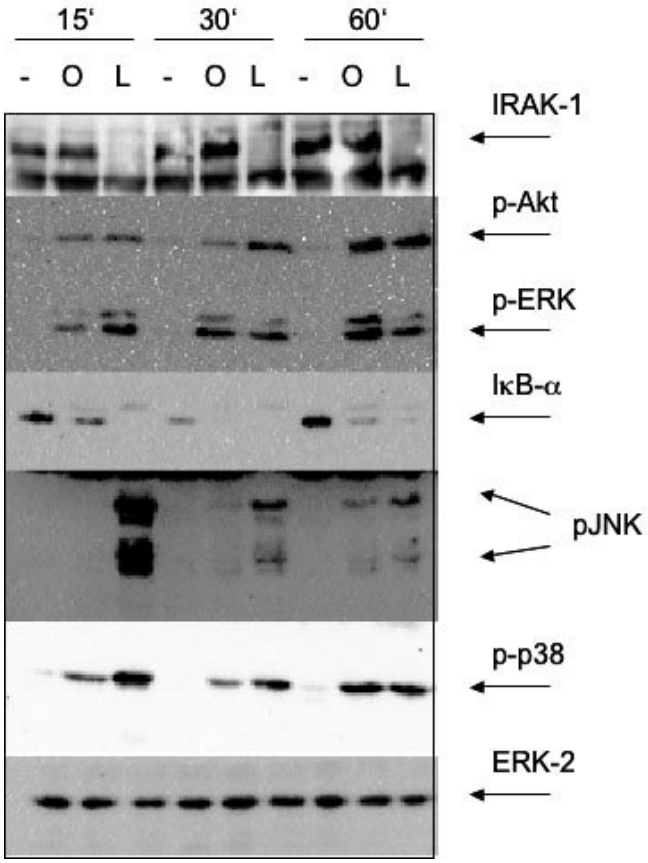

Figure 6. OPN induces signal transduction in macrophages. M1 macrophages were treated with 1 µg/ml OPN (O), 100 ng/ml LPS (L), or left untreated for indicated time periods. Cell lysates were prepared and immunoblots probed with antibodies against respective proteins, ERK-2 as loading control. A representative experiment out of three is shown.

To further verify this observation, we measured JNK phosphorylation by intracellular staining and flow cytometry. As shown in Figure 7, LPS led to a 2-fold increase of pJNK-positive cells (unstimulated: 14.9% ± 2.5%; 15' stimulated: 32% ± 3.1%, P = 0.004). OPN did not lead to a profound increase in pJNK-positive cells (21.2% ± 3.8%), thereby indicating, consistent with Western Blot results, that OPN does not significantly activate the JNK/c-JUN pathway in macrophages.

In summary, these data suggest that OPN is activating MAP Kinase pathways and the Akt pathway, as well as the NfκB pathway to a minor extent in human macrophages.

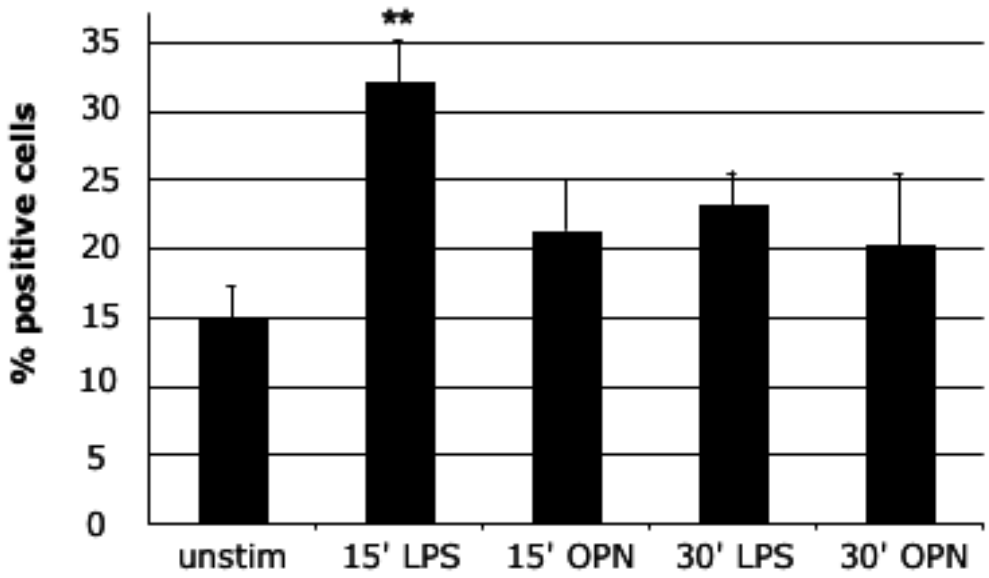

Figure 7. Phosphorylation of JNK in M1 macrophages. Cells were stimulated with 1 µg/ml OPN, 100 ng/ml LPS, for indicated time periods, or left untreated, fixed and permeabilized and stained intracellularly for pJNK. Activation of pJNK was assessed by flow cytometry and depicted as percentage of pJNK-positive cells (n=13). Data are presented as mean ± SE. $^{**}P < 0.01$ vs unstimulated.

4.1.2 OPN-induced cytokine production of M1 and M2 macrophages

ATMs are of M2-like phenotype, hence in our experiments we are using M2 (IL-4-induced) macrophages as a model for ATM cytokine production in addition to conventional M1. Preliminary experiments had shown that LPS activation of M2 leads to production of substantial amounts of the anti-inflammatory cytokine IL-10 and the chemokine monocyte chemoattractant protein-1 (Mcp-1), which is implicated in inflammatory processes of obese AT. In contrast, stimulation with OPN led to the production of high amounts of the inflammatory Mcp-1 but did not induce the anti-inflammatory IL-10.

To investigate concentration-dependent effects and putative interactions of OPN with LPS-induced activation, we stimulated M1 and M2 macrophages with indicated combinations of OPN and LPS concentrations. After 2 days, Mcp-1, IL-10 and TNFα concentrations in the supernatants were measured. In M2 macrophages, OPN induced moderate TNFα production in a concentration-dependent manner (130.5 ± 74 pg/ml, Figure 8a), whereas essentially no IL-10 and high amounts of Mcp-1 (6968.7 ± 2119.7 pg/ml, $P = 0.026$, Figure 8b,c) were produced. However, neither an enhancement of

LPS-induced inflammatory cytokine production nor a suppression of LPS-induced anti-inflammatory reactions was observed.

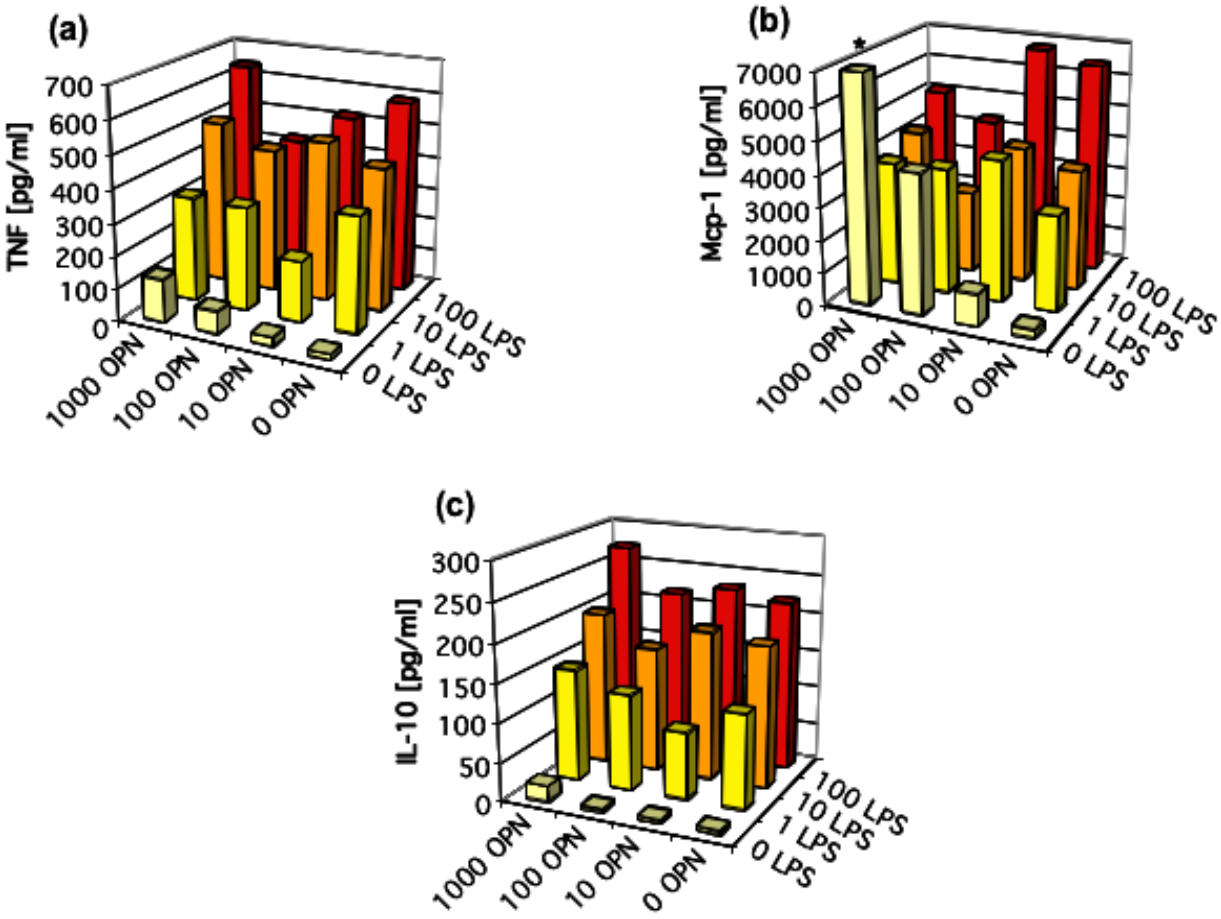

Figure 8. OPN-induced cytokine production of M2 macrophages. M2 were stimulated with indicated concentrations (ng/ml) of OPN and LPS for 2 days and TNFα (a), Mcp-1 (b), and IL-10 (c) concentrations in the supernatants were measured. Diagrams show the mean concentrations of 2 donors. **P* < 0.05 compared to untreated.

In M1 macrophages, Mcp-1 production was similar to M2 (20,178 ± 3,114.5 pg/ml *P* = 0,009, Figure 9b). TNFα was only induced by the highest concentration of OPN alone (419.1 ± 132.4 pg/ml, *P* = 0.038), but LPS-induced TNFα production was further enhanced by OPN (LPS: 922.794 ± 299 pg/ml; LPS+OPN: 1,271 ± 436 pg/ml, Figure 9a). In contrast to the results obtained for M2, OPN was able to induce IL-10 production in M1 synergistically with LPS (OPN: 121 ± 43 pg/ml; OPN+LPS: 289 ± 76 pg/ml, Figure 9c).

These data indicate that OPN is able to induce pro- and anti-inflammatory cytokine

production in human macrophages, whereas the exact pattern of secreted cytokines depends on the particular model system.

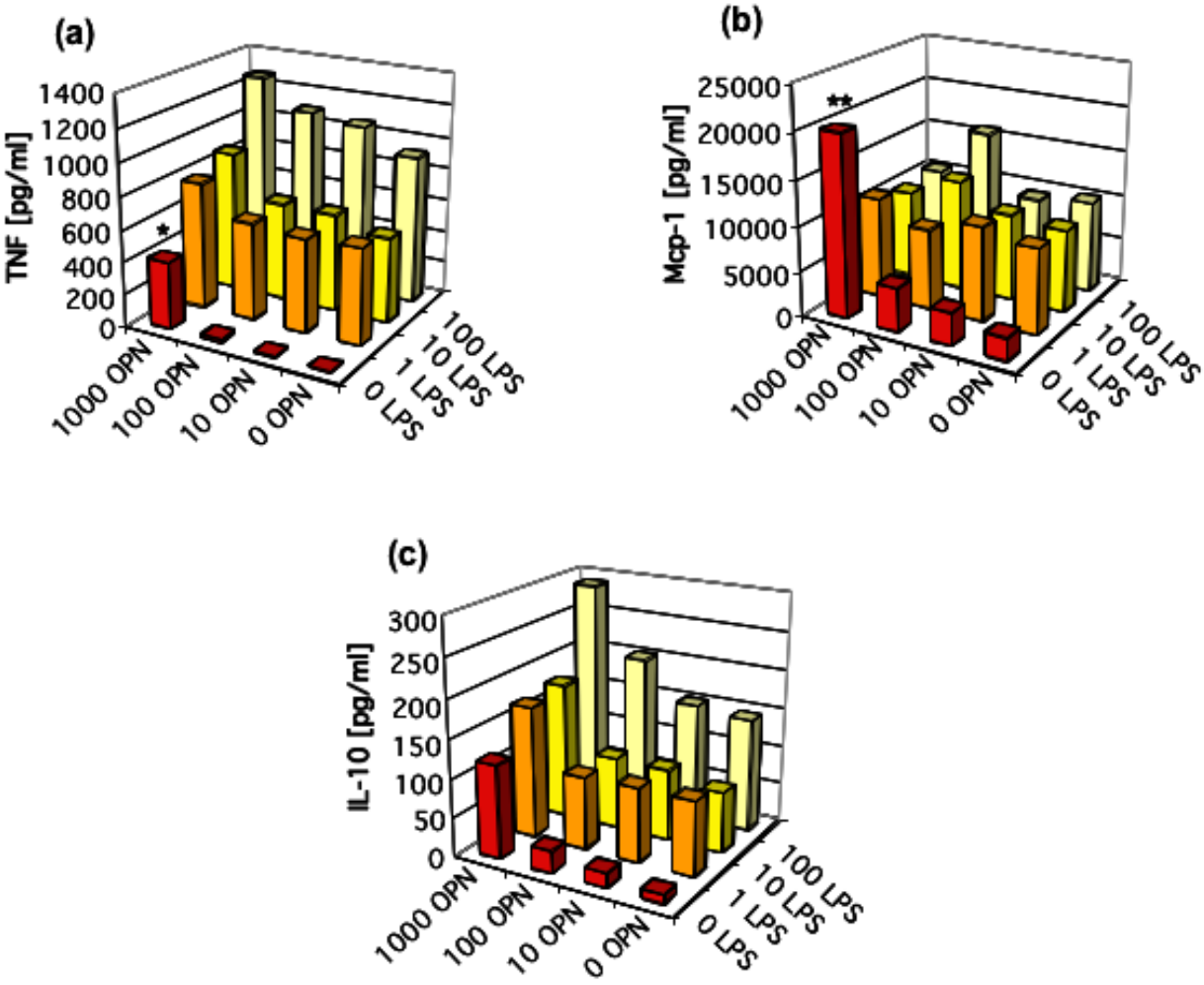

Figure 9. OPN-induced cytokine production of M1 macrophages. M1 were stimulated with indicated concentrations (ng/ml) of OPN and LPS for 2 days and Mcp-1 (a), TNFα (b) and IL-10 (c) concentrations in the supernatants were measured. Diagrams show the mean concentrations of 2 donors. *$P < 0.05$, **$P < 0.01$ compared to untreated.

4.1.3 Cytokine secretion of human ATMs

4.1.3.1 Cytokine production of magnetically sorted $CD14^+$ AT-SVC

ATMs have been characterized by the expression of CD14 and MR on their surface (118) and several lines of evidence previously suggested that $CD14^+MR^-$ cells prepared

from AT indicate contamination with blood monocytes probably from microvessels. Here, we isolated $CD14^+$ cells from human adipose tissue via magnetic cell sorting, which is a fast and easy procedure but yields a cell population containing a considerable number of blood monocytes (~20% of the total $CD14^+$ population, data not shown). To determine the inflammatory mediator secretion pattern of ATMs, we stimulated $CD14^+$ cells from subcutaneous AT with OPN, LPS, or IFNγ and analyzed cytokine secretion into the culture medium and also the CD14-depleted SVC (dSVC) fraction was included in this analysis. The investigated cytokines except Mcp-1 (601 ± 399.1 pg/ml) were not produced by the dSVC fraction in significant amounts. Therefore, production of the other cytokines by $CD14^-$ cells can be disregarded. We observed that $CD14^+$ cell preparations produced large amounts of TNFα (390 ± 77 pg/ml) and Mcp-1 (1,141 ± 111 pg/ml) and moderate amounts of the anti-inflammatory IL-10 (199 ± 82 pg/ml). Basal expression of all cytokines was markedly higher than in M1 and M2 model macrophages. Remarkably, $CD14^+$ cells were only weakly stimulated by LPS, but responded to IFNγ with increased TNFα (968 ± 310 pg/ml) and Mcp-1 (2580 ± 559 pg/ml) secretion. Moreover, cells did essentially not respond to OPN, except for a moderate increase in Mcp-1 production (2005 ± 1092 pg/ml), whereas a possible contribution of contaminating $CD14^-$ cells thereto cannot be excluded (Figure 10).

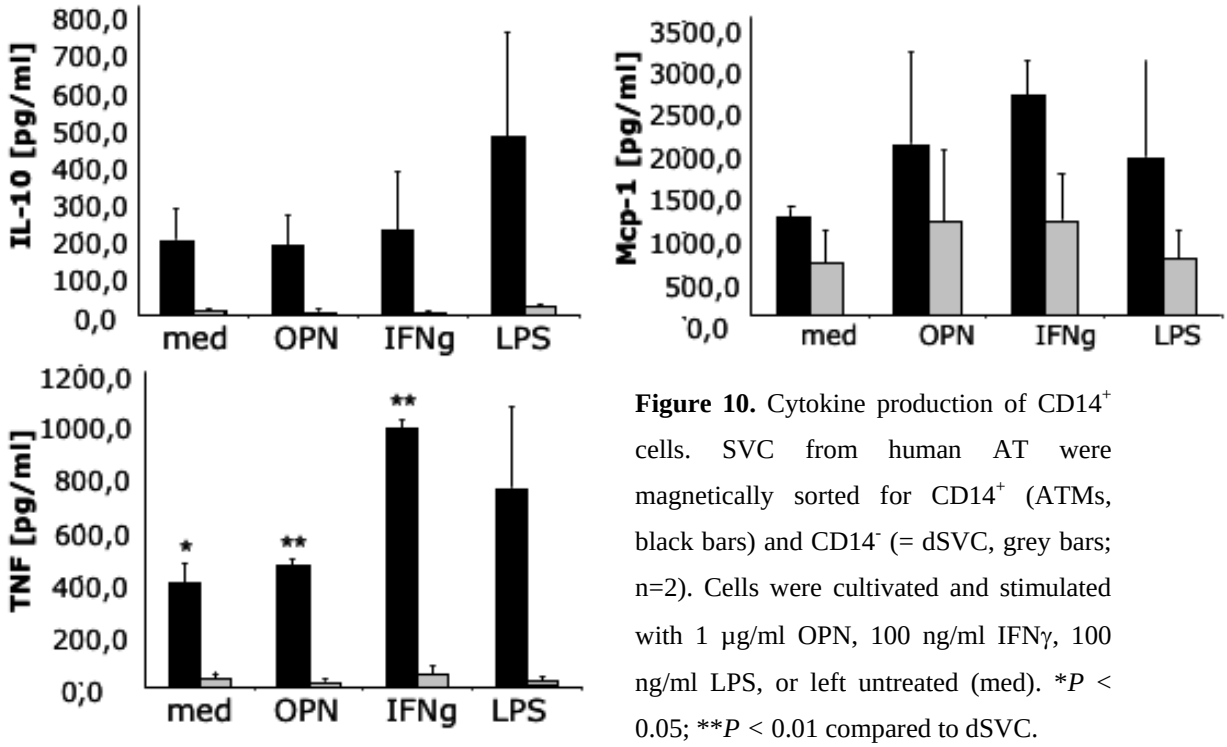

Figure 10. Cytokine production of $CD14^+$ cells. SVC from human AT were magnetically sorted for $CD14^+$ (ATMs, black bars) and $CD14^-$ (= dSVC, grey bars; n=2). Cells were cultivated and stimulated with 1 µg/ml OPN, 100 ng/ml IFNγ, 100 ng/ml LPS, or left untreated (med). **P* < 0.05; ***P* < 0.01 compared to dSVC.

4.1.3.2 Cytokine expression of $CD14^+MR^+$ ATMs

Since magnetically sorted $CD14^+$ ATMs are contaminated with blood monocytes, we used flow cytometry to separate $CD14^+MR^+$ ATMs from $CD14^+MR^-$ cells. This procedure provided not only another method of ATM purification, but also enabled us to detect potential cytokine production of $CD14^+MR^-$ cells that may contribute to the results obtained for magnetically sorted $CD14^+$ cells.

The initial populations usually contained ~ 4% $CD14^+MR^-$ and ~ 15% $CD14^+MR^+$ cells. Sorted populations had approximately 75% purity, the other 25% predominantly consisting of $CD14^-$ cells and cell fragments that may not disturb cultivation experiments (Figure 11).

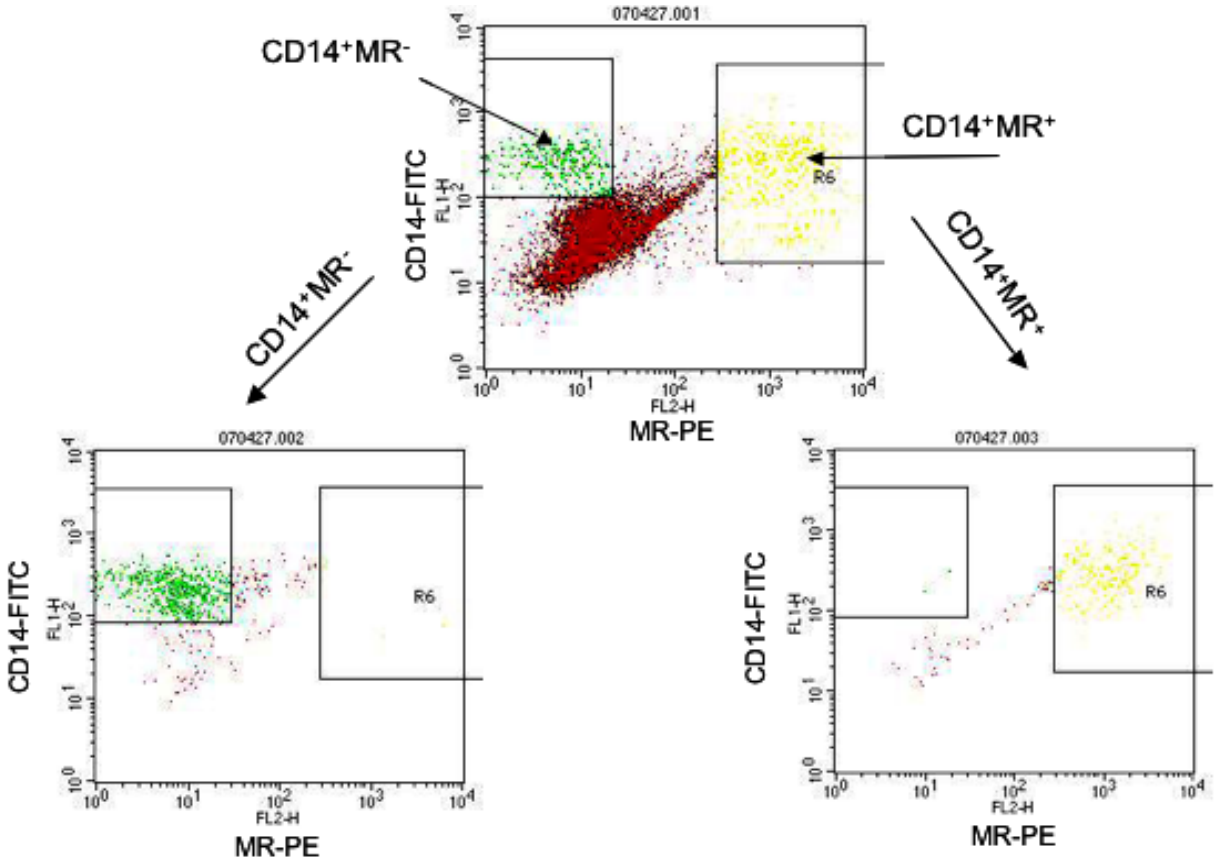

Figure 11. Isolation of $CD14^+MR^*$ ATMs. AT SVC were stained with CD14-FITC and MR-PE antibodies and sorted by FACS. Cell populations prior (top) and post (bottom) sorting are shown in a representative dot blot.

Isolated MR^+ and MR^- cells were cultured separately and treated with OPN, IFNγ, LPS,

or left untreated. As shown in Figure 12, basal concentration of Mcp-1 was similar to $CD14^+$ cells (932 ± 31.4 pg/ml) whereas IL-10 and TNFα were hardly detected in untreated FACS-sorted ATMs. Remarkably, basal cytokine concentrations were equal in MR^+ and MR^- cells and both populations did not respond to IFNγ treatment, which is conflicting with the results obtained for $CD14^+$ cells. Moreover, both OPN and LPS induced moderate IL-10 (OPN: 749 ± 245 pg/ml; LPS: 491 ± 167 pg/ml) and TNFα production (OPN: 39 ± 1 pg/ml, $P < 0.001$; LPS: 27 ± 2.6 pg/ml, $P = 0.001$) and Mcp-1 was not induced by any of the substances except OPN in MR^- cells only (4,413 ± 3,586 pg/ml).

These results suggest that contaminating $CD14^+MR^-$ monocytes are also capable of cytokine production and may be activated further, which should be considered when investigating $CD14^+$ magnetically sorted cells. However, there were profound differences in activation capacity and cytokine expression patterns of ATMs isolated by either MACS or FACS.

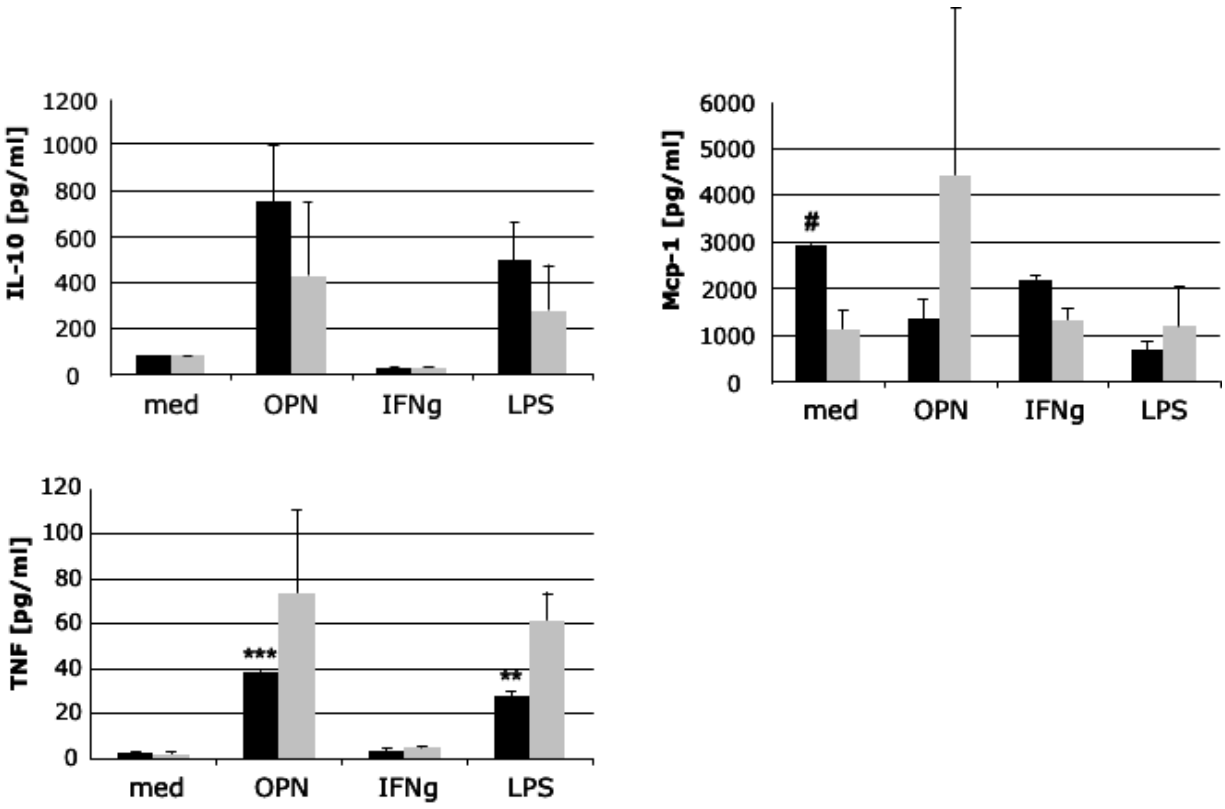

Figure 12. Cytokine production of ATMs. Human SVC were stained for CD14 and MR and sorted by FACS into a $CD14^+MR^+$ (black bars) and a $CD14^+MR^-$ (grey bars) population (n=2). Both populations were cultivated and stimulated with 1 µg/ml OPN, 100 ng/ml IFNγ, 100 ng/ml LPS, or left unstimulated (med). After 3 days, concentrations of indicated cytokines were measured in the supernatants. Data are presented as mean ± SE. $^{**}P < 0.01$, $^{***}P < 0.001$ versus medium; $\#P < 0.05$ versus MR^-.

4.1.4 Gene expression of human ATMs

Several lines of evidence suggest that in humans, only $CD14^+MR^+$ cells can be referred to as ATMs (118). OPN is an inflammatory cytokine that is extensively produced by activated macrophages (119). Thus, we hypothesized that predominantly $CD14^+MR^+$ cells mediate inflammatory cytokine production, including OPN, of the adipose tissue. Nevertheless, evidence to support this theory has been lacking so far and a potential role of $CD14^+MR^-$ cells in AT inflammation cannot be excluded, either. Therefore, we isolated $CD14^+MR^+$ and $CD14^+MR^-$ populations from human subcutaneous (n = 3) and omental (n = 6) AT SVC and examined cytokine and OPN gene expression by RT-qPCR. We observed that all of the investigated cytokines, including OPN, IL-10, Mcp-1, and TNFα where significantly higher expressed in MR^+ compared to MR^- cells (OPN: 8-fold increase, P = 0,042; IL-10: 2-fold increase, P = 0,0097; Mcp-1: 6-fold increase, P = 0,016; TNFα: 4-fold increase, P =0,018; Figure 13). These data indicate that human $CD14^+MR^+$ ATMs are capable of excessive OPN and inflammatory cytokine production, thereby confirming that MR^+ and not MR^- cells predominantly contribute to obesity-related AT inflammation.

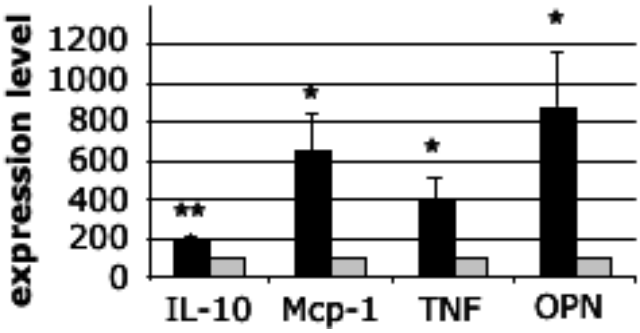

Figure 13. Gene expression of human ATMs. Human SVC were sorted into a $CD14^+MR^+$ (black bars, n=9) and a $CD14^+MR^-$ (grey bars, n=9) population, and mRNA levels of indicated genes were measured by RT-qPCR. Gene expression levels were compared to the MR^- population, which was designated as 100%. Data are presented as mean ± SE. $*P < 0.05$, $**P < 0.01$ compared to MR^-.

4.1.5 ATM surface markers are not altered in *spp1* knockout mice

Preliminary experiments of our group revealed no significant difference in ATM

number between wt and *spp1* knockout mice. We therefore aimed to investigate whether OPN deficiency has an influence on the activation state of ATMs by characterizing the expression of the ATM activation marker CD11c (116), the M2 marker MR and additionally, the OPN receptors CD51 and CD44 in wt and *spp1* knockout ATMs.

Epididymal fat tissue plays a significant role in overall metabolism in rodents (139). We thus isolated SVC from epididymal fat pads excised from male *spp1* knockout or wt mice fed a LFD or HFD and analyzed cells by flow cytometry. Investigated surface markers included F4/80, and, as mentioned above, CD11c, MR, and the putative OPN receptors CD51 and CD44. F4/80 has been used by most previous studies as a single marker to identify murine ATMs. As expected, we observed an increase of $F4/80^+$ cells in HFD mice (26.1% ± 1.3% of SVC) compared to LFD mice (17% ± 2.7% of SVC), and correlating with our previous observation there was no difference between the wt and the *spp1* genotype (*spp1:* 26.4% ± 2.5% HFD; 19.3% ± 3.2% LFD, Figure 14a), which was additionally confirmed by immunohistochemistry (data not shown).

We observed that in LFD mice, all $F4/80^+$ cells were also MR^+, compared to only 65% MR^+ of $F4/80^+$ in HF mice (wt: 62.5% ± 3.9%, $P = 0.0019$; *spp1*: 69.5% ± 3.9%, $P = 0.00047$, Figure 14d) thereby suggesting the appearance of a $F4/80^+MR^-$ cell population upon high-fat feeding. According to a recent publication, $CD11c^+$ macrophages accumulate in the AT of diet-induced obese mice (116). In our experiments, the percentage of $F4/80^+CD11c^+$ macrophages in HFD (wt: 33.3% ± 0.9%; *spp1:* 31.7% ± 3.7%) was three fold higher than that of LFD control mice (wt: 9.5% ± 2.3%, $P = 0.00023$; *spp1:* 5.6% ± 2.2%, $P = 0.0032$, Figure 14e) confirming results obtained by Lumeng et al (116). Moreover, cells were gated for MR^+ cells and analyzed for CD11c coexpression. We observed that in the MR^+ population, CD11c was equally elevated as in the total $F4/80^+$ cell population (HFD: wt: 33.3 ± 0.9%, *spp1:* 31% ± 3.8%; LFD: wt: 13.5% ± 2%, $P < 0.00001$; *spp1:* 10.5% ± 1.5%, $P = 0.013$, Figure 14f). Finally, expression of CD51 and CD44 was significantly upregulated in HFD mice (CD51: wt: 539.4 ± 25.4; *spp1*: 455.9 ± 64.7; CD44: wt: 2043.8 ± 155.4; *spp1:* 1877.4 ± 300.6) compared to LFD mice (CD51: wt: 140.7 ± 18.5, $P < 0.0001$; *spp1*: 147 ± 21.1, $P = 0.03$; CD44: wt: 782.7 ± 155.4, $P = 0.0044$; *spp1:* 684.3 ± 34.9, $P = 0.036$; Figure 14i, j)

However, none of the investigated surface markers was altered in *spp1* knockout mice compared to wt mice, indicating that there are no alterations in ATM number, OPN receptor, and activation marker expression upon OPN deficiency.

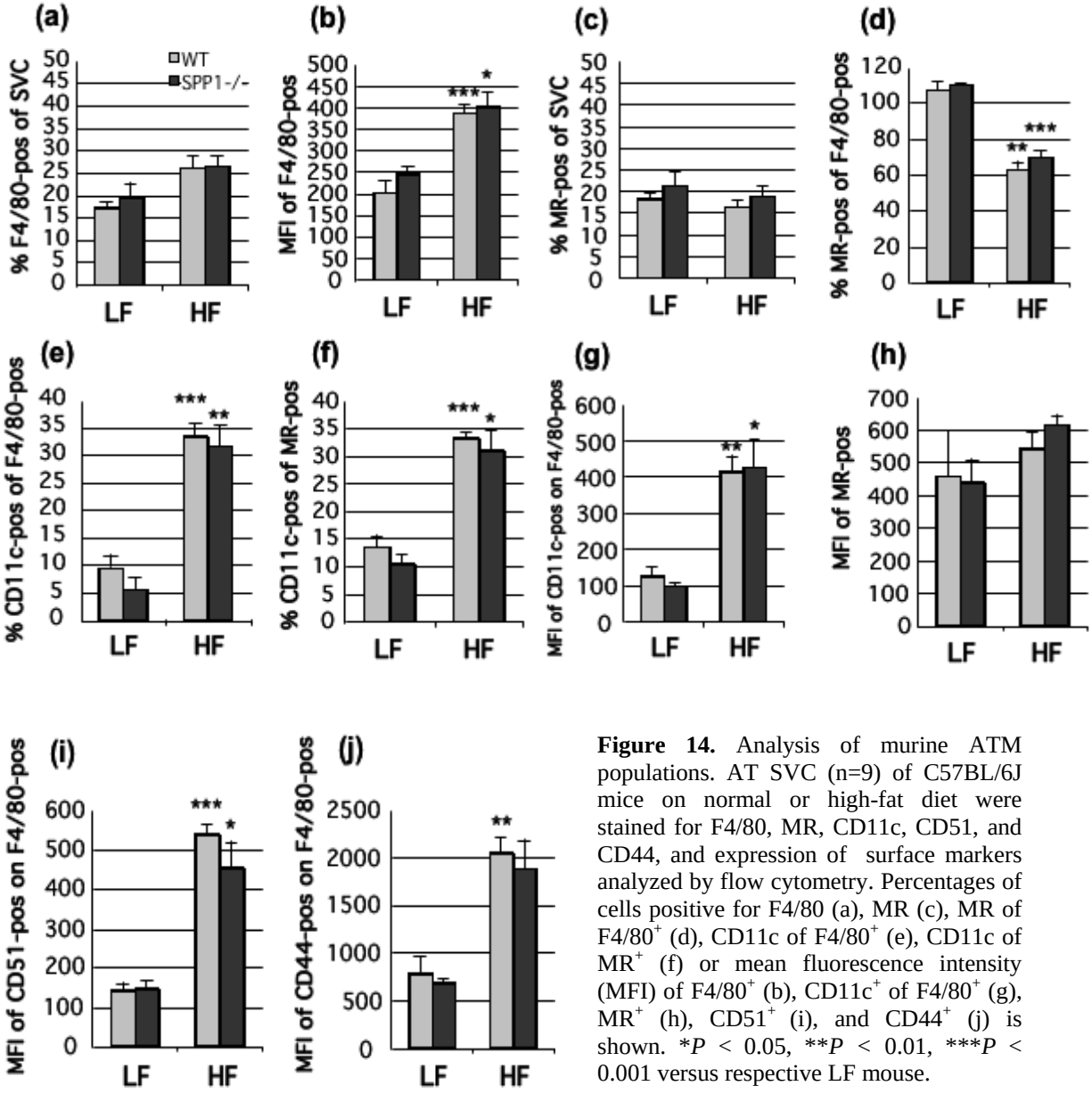

Figure 14. Analysis of murine ATM populations. AT SVC (n=9) of C57BL/6J mice on normal or high-fat diet were stained for F4/80, MR, CD11c, CD51, and CD44, and expression of surface markers analyzed by flow cytometry. Percentages of cells positive for F4/80 (a), MR (c), MR of F4/80$^+$ (d), CD11c of F4/80$^+$ (e), CD11c of MR$^+$ (f) or mean fluorescence intensity (MFI) of F4/80$^+$ (b), CD11c$^+$ of F4/80$^+$ (g), MR$^+$ (h), CD51$^+$ (i), and CD44$^+$ (j) is shown. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus respective LF mouse.

4.2 The effect of OPN on adipocytes

Spp1 knockout mice display improved insulin sensitivity compared to wt mice. In order to evaluate a possible influence of OPN on adipocyte insulin responsiveness, we treated murine 3T3-L1 and human adipocytes with OPN for 48 h. As a control, cells were treated with TNFα which has repeatedly been shown to inhibit insulin-induced glucose uptake in adipocytes (116, 140). Insulin sensitivity was assessed by insulin-stimulated 2-deoxyglucose uptake. We observed that OPN did not affect glucose uptake in 3T3-L1 cells, and only a slight inhibition of insulin sensitivity was observed in human adipocytes (*P* = 0.056). TNFα effectively interfered with insulin-induced glucose

uptake in both model systems (Figure 15).

Impaired adipocyte differentiation results in malfunctioning adipocytes with reduced lipid accumulation and insulin-induced glucose transport capabilities. Previous studies have suggested that PPARγ expression, which is induced during adipocyte differentiation, suppresses OPN transcription in macrophages (141) and adipocytes (129), leading to decreased OPN expression during adipocyte differentiation. Thus, we investigated the effect of OPN on adipocyte differentiation. Human preadipocytes were differentiated with or without the addition of OPN to the medium. At differentiation end points, cells were examined morphologically and gene expression of adipocyte differentiation markers as well as adiponectin was measured via RT-qPCR. Morphological examination revealed no difference between OPN-treated and untreated adipocytes (Figure 16), yet there was a trend of a decrease in the expression of the adipocyte differentiation markers PPARγ (82% ± 11.9%, $P = 0.14$) and GLUT4 (82% ± 14.4%, $P = 0.23$, Figure 17). Adiponectin is associated with increased adipocyte differentiation capacity and insulin sensitivity and its expression was shown to be decreased in obesity. Here we observed that adiponectin expression is decreased in adipocytes chronically treated with OPN (75% ± 6.3%, $P = 0.0034$, Figure 17).

To conclude, these results indicate that OPN has no profound effect on insulin sensitivity, but a moderate effect on adipocyte differentiation capacity and adipoQ expression.

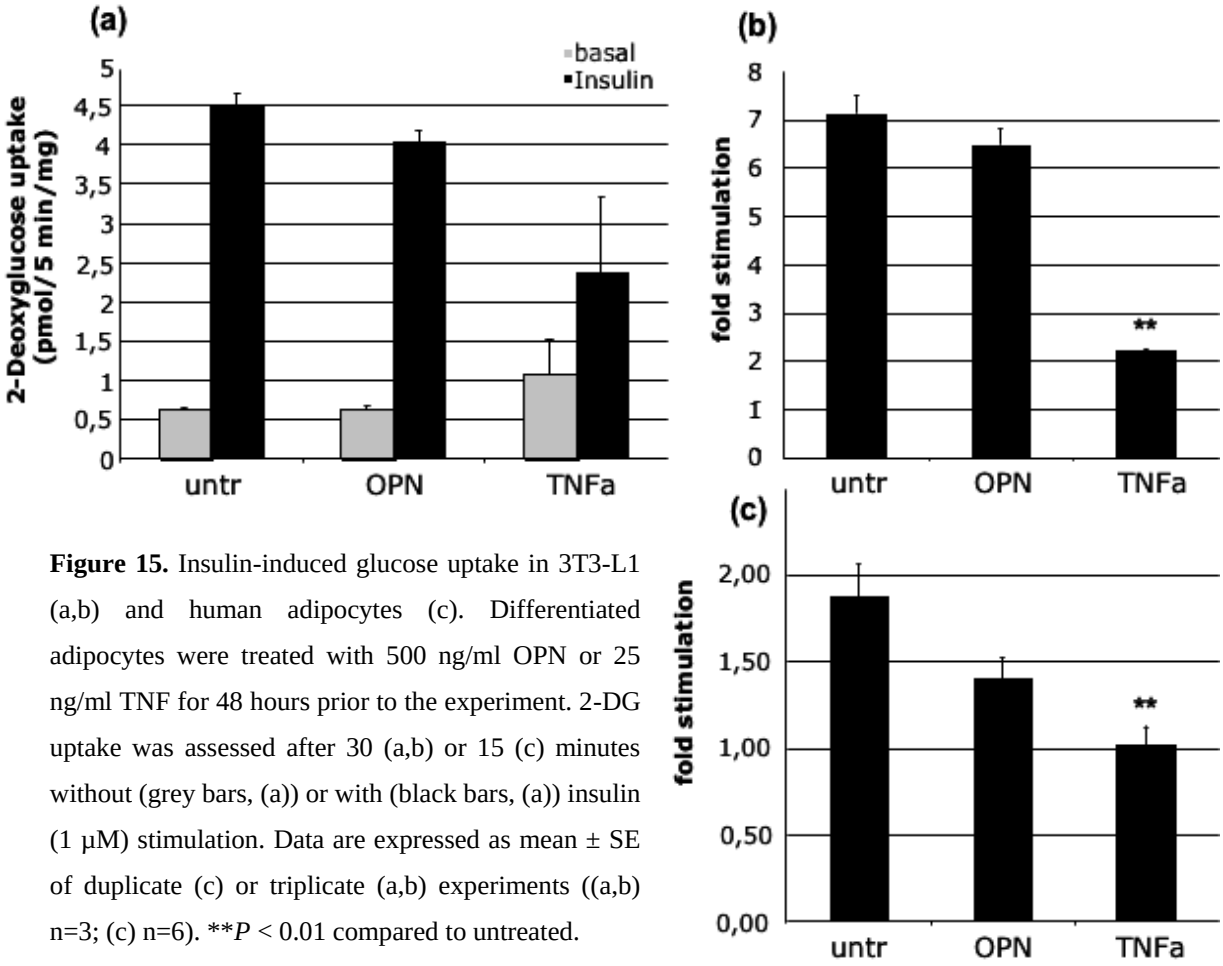

Figure 15. Insulin-induced glucose uptake in 3T3-L1 (a,b) and human adipocytes (c). Differentiated adipocytes were treated with 500 ng/ml OPN or 25 ng/ml TNF for 48 hours prior to the experiment. 2-DG uptake was assessed after 30 (a,b) or 15 (c) minutes without (grey bars, (a)) or with (black bars, (a)) insulin (1 µM) stimulation. Data are expressed as mean ± SE of duplicate (c) or triplicate (a,b) experiments ((a,b) n=3; (c) n=6). $^{**}P < 0.01$ compared to untreated.

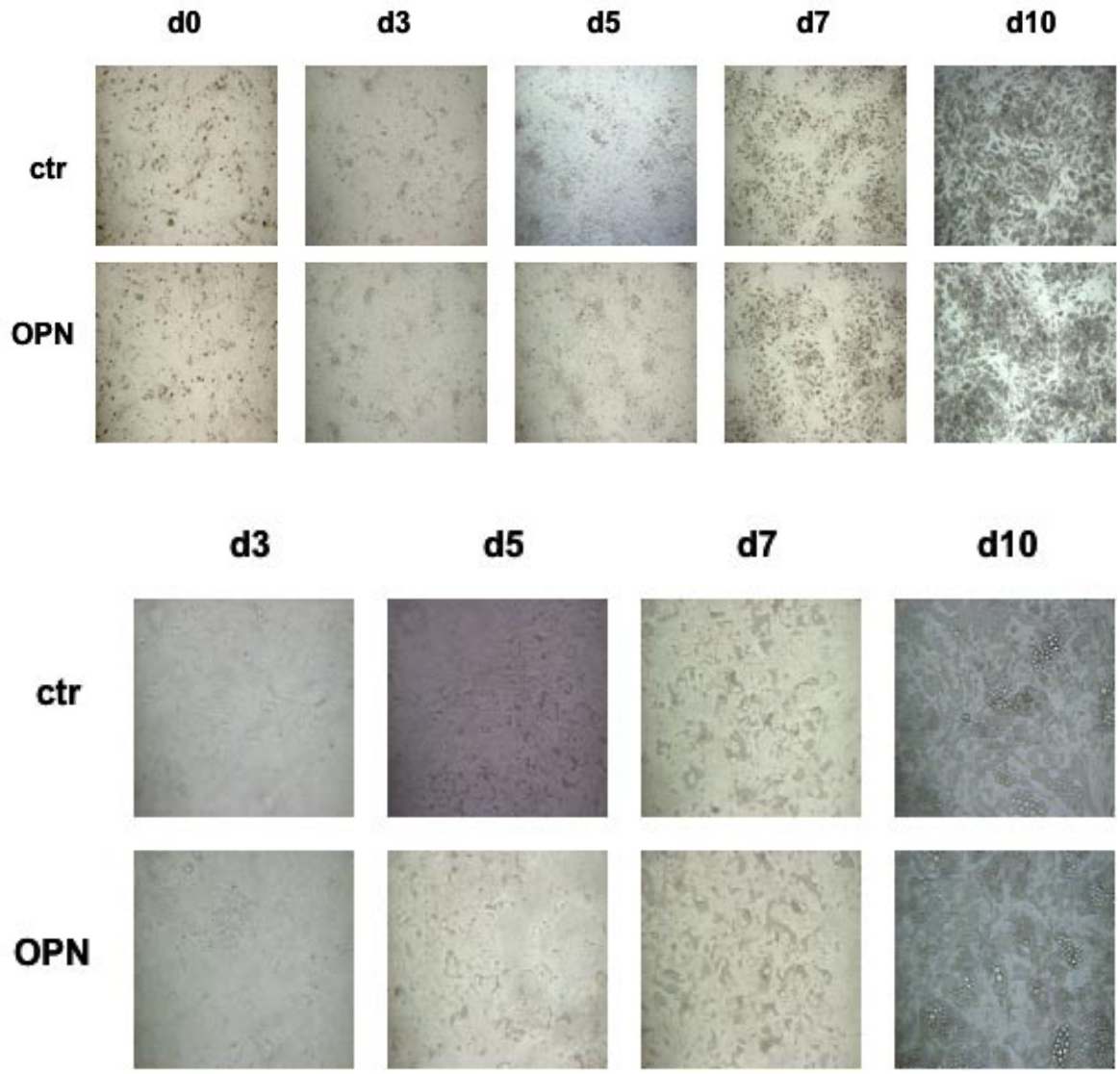

Figure 16. Morphology of human preadipocytes differentiating with or without 500 ng/ml OPN. (magnification: x100 (top), x 1,000 (bottom).

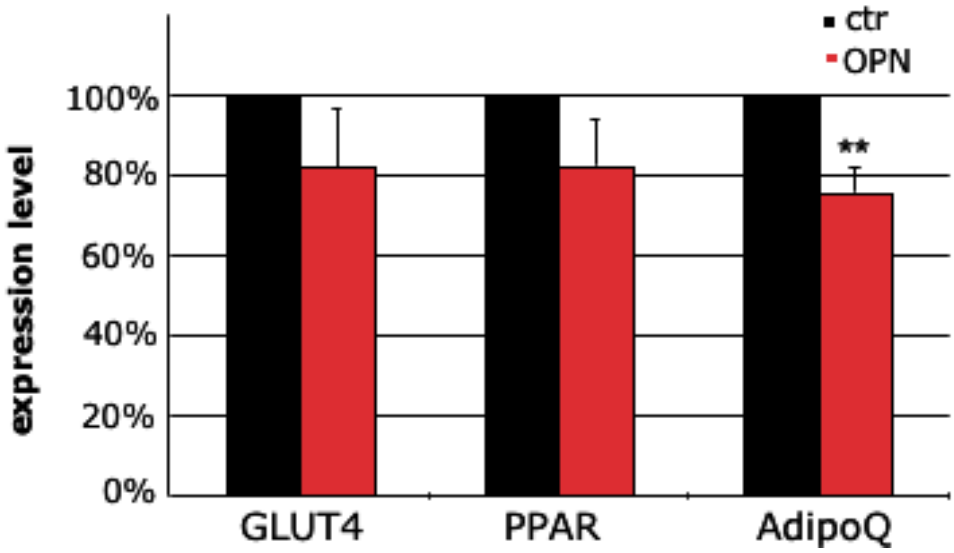

Figure 17. Adipocyte marker expression in OPN-treated human adipocytes. Human preadipocytes were differentiated with (red bars) or without (black bars) the addition of 500 ng/ml OPN to the culture medium. At d10 of differentiation, cells were harvested and gene expression of GLUT4, PPARγ and adipoQ quantitatively assessed by RT-qPCR (n=4). Data are presented as relative gene expression normalized to 18S rRNA gene expression and are expressed as mean ± SE. $^{**}P < 0.01$, compared to control.

4.3 Coculture of spp1 and wt macrophages with 3T3-L1 adipocytes

Our results indicate that OPN is an activator of ATMs but does not affect adipocyte function. In a previous publication, macrophages have been shown to interfere with adipocyte insulin sensitivity in coculture experiments (113). We hypothesized that *spp1* knockout macrophages may have lost this ability if OPN is crucial for macrophage activation in AT inflammation. In order to address this question, we employed indirect coculture experiments with AT SVC derived from obese wt C57BL/6J or *spp1* knockout mice.

Adherent SVC (10^5 cells/insert) were cultured in the upper chamber of a permeable cell culture insert with 3T3-L1 adipocytes in the lower chamber. The permeable membrane in this culture system permits secreted factors to pass between the two cell types but does not permit direct cell – cell contact. As shown in Figure 18, coculture with both wt and *spp1* SVC led to an increase in basal glucose uptake, which decreased the fold change in insulin-stimulated glucose uptake. There was no difference between wt and *spp1* macrophage-mediated inhibition of insulin response indicating that macrophage-inherent OPN is not essential for interference with adipocyte function.

Since OPN is an activator of ATMs, we sought to examine whether treatment of macrophage – adipocyte cocultures with OPN had any effect on adipocyte insulin resoponsiveness compared to nontreated cocultures. Therefore, SVC from wt mice fed a normal diet (ND) were isolated and cocultured with adipocytes as described above, and treated with OPN or left untreated as a negative control. Similar to the HFD-SVC, ND-SVC led to an increased basal glucose uptake of 3T3-L1 adipocytes, thereby resulting in a decreased fold stimulation index of insulin-induced glucose uptake. However, there was no difference between OPN-treated and untreated coculture systems.

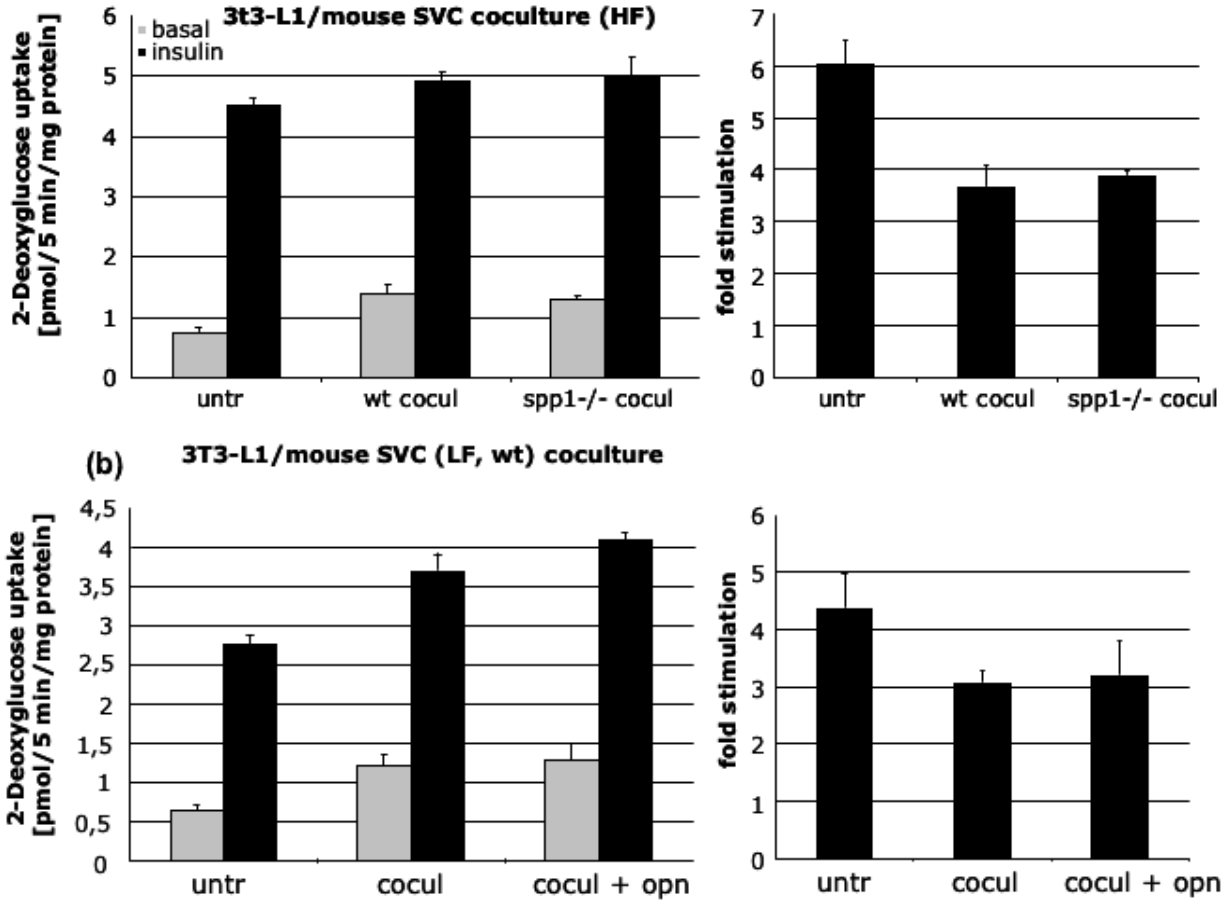

Figure 18. Insulin-stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes cocultured with murine SVC. (a) Murine SVC obtained from HFD wt and *spp1* mice (n=4) and (b) murine SVC obtained from lean wt mice were cultured in the upper cell culture insert with differentiated 3T3-L1 adipocytes in the lower chamber for two days. 2-DG uptake was assessed with and without insulin stimulation. Experiments were performed in duplicate (a) or triplicate (b) and data are expressed as means ± SE.

5 Discussion

Several lines of evidence have clearly demonstrated that obesity is accompanied by a state of chronic, low-grade inflammation that contributes to insulin resistance and type 2 diabetes (10). It has also been shown that obesity leads to macrophage accumulation in AT and it is likely that macrophages are the main contributors to the inflammatory responses observed in obesity (34, 60). OPN is a chemokine-like, extracellular matrix-associated protein involved in monocyte motility and inflammatory immune responses (130). According to two recent studies (129, 131), OPN expression is increased in the AT of obese mice and OPN deficiency leads to improved insulin sensitivity as well as decreased AT inflammation (129). It was the aim of this thesis to investigate the influence of OPN on ATMs and adipocytes in order to elucidate the mechanisms by which OPN contributes to insulin resistance.

A crucial role of OPN in the attraction and activation of murine ATMs has already been proposed (129). Therefore we focused our experiments on human model systems. We report here that OPN is a potent activator of human macrophages.

Human ATMs were previously characterized by CD14 and MR coexpression, whereas $CD14^+MR^-$ cells found in obese AT were thought to be contaminating blood monocytes (118). Consistent with this notion, we show here that on the mRNA level, $CD14^+MR^+$ ATMs express larger quantities of OPN and other cytokines when compared to $CD14^+MR^-$ monocytes. Hence, in this study $CD14^+MR^+$ cells were regarded as ATMs. Isolation of sufficient amounts of human ATMs is crucial for investigating their nature and function. We applied here two different methods of ATM purification, magnetic cell sorting (MACS) and flow cytometry, and compared their responsiveness to OPN and other cell stimulators in terms of cytokine secretion. Magnetic cell sorting is a fast and easy procedure for the enrichment of cell populations via specific surface molecules. However, if analyses of ATM function are required, only anti-CD14 magnetic beads can be used since ATMs magnetically isolated via MR have been shown not to be functionally intact (own unpublished observations). Therefore, conclusions from experiments with MACS-separated ATMs are limited, because ATMs isolated that way contain considerable numbers (~ 20%) of MR^- contaminating blood monocytes.

MACS-sorted $CD14^+$ cells basally secreted IL-10, Mcp-1 and TNFα, and were

stimulated by IFNγ to further increase Mcp-1 and TNFα, whereas OPN had little effect. Remarkably, the CD14-depleted fraction also produced considerable amounts of Mcp-1 with a tendency to be stimulated further. This phenomenon may point towards an important role of $CD14^-$ cells of the AT (preadipocytes, endothelial cells) in the attraction of macrophages.

Flow cytometry yields a much higher purity of ATMs, since sorting via both CD14 and MR is possible, but there were remarkable differences in cytokine production compared to MACS-sorted cells. First, $CD14^+MR^+$ cells secreted hardly any IL-10 and TNFα on the basal level. Moreover, they had only very little potential to be activated further: IL-10 was the only cytokine that could be induced by OPN or LPS, and IFNγ had, in contrast to MACS-sorted cells, no effect at all.

Also, the $CD14^+MR^-$ sorted fraction secreted hardly any IL-10 or TNFα and basal Mcp-1 secretion was lower in MR^- than in MR^+ cells, correlating with the gene expression results. However, the MR^- population had a higher capability of being stimulated than the MR^+ population. If MR^+ cells are referred to as „real“ ATMs and MR^- cells are considered as contaminating blood monocytes, this may appear reasonable, since $CD14^+MR^+$ cells are probably preactivated by inflammatory processes in the AT and therefore show limited capacity of being activated further compared to $CD14^+MR^-$ cells that were not preactivated.

Nevertheless, this also suggests that cytokine production of stimulated MACS-sorted $CD14^+$ cells may be, at least in part, attributed to MR^- non-ATMs. Because of the discrepancies observed in cytokine production of the two differently sorted ATMs, a major resulting question is, which purification method is to be preferred. Whereas MACS-sorted $CD14^+$ cells contain approximately 20% MR^- contaminating cells, FACS-sorted ATMs are of high purity but have obviously lost their capacity of being stimulated. However, it can be assumed that either method, especially in combination with subsequent cultivation, leads to alterations in cell function that does not necessarily reflect the in vivo situation.

Since it is difficult to obtain high amounts of ATMs by the mentioned methods, we used human model macrophages in our further experiments. We showed that OPN leads in a concentration-dependent manner to the production of the inflammatory mediators TNFα and Mcp-1, and the anti-inflammatory cytokine IL-10. The exact cytokine secretion pattern is dependent on the type of model macrophage activation: Whereas

M2 macrophages do not produce anti-inflammatory IL-10 upon OPN-stimulation and do not show any OPN-LPS synergism, M1 macrophages do so. Although M2 macrophages resemble ATMs phenotypically more than M1 macrophages, ATMs cannot definitely be assigned to the one or the other type, since M1 and M2 are only two extremes of a continuum of functional stages of macrophages (103). At any rate, the results demonstrate clearly that OPN leads to the production of inflammatory mediators in model macrophages as well as ATMs. Exceptionally high is the production of Mcp-1 in response to OPN which suggests that a main role of OPN in AT inflammation could be the attraction of immune cells via induction of chemokines. The production of the anti-inflammatory cytokine IL-10, which has been shown to have beneficial effects on insulin sensitivity (116), indicates that the response to OPN is not exclusively inflammatory, though.

On the signaling level, we found that OPN signals independently from IRAK-1 degradation, which is a central event in LPS-mediated TLR4 signaling. This finding is important since previously doubts have been raised that results on OPN-induced immune cell activation may be due to LPS contamination of OPN preparations (128). Moreover, OPN induces activation of MAP Kinase pathways and, to a minor extent, activation of the NfκB pathway, whereas analyses of time courses showed that OPN signals somewhat delayed compared to the classical cell stimulator LPS.

Since according to our data, OPN deficiency reduces AT inflammation without affecting macrophage cell number, we aimed to investigate an influence of OPN on ATM phenotype as detected by CD11c, MR, and OPN receptor expression. However, investigated surface markers were not altered in ATMs of *spp1* knockout compared to wt mice. We detected considerable upregulations of CD11c, an AT inflammation marker, and the putative OPN receptors CD51 and CD44 in high-fat diet fed mice. In further experiments, it would be interesting to investigate which of these ATM populations is the OPN-expressing one. We moreover found that the percentage of MR^+ of $F4/80^+$ decreases after HF feeding, indicating the emergence of a novel $F4/80^+MR^-$ cell population also independent of OPN. We cannot interpret the importance of this population at the moment, but further experiments will reveal potential differences in gene expression or cytokine production between these two cell types. Macrophage downregulation of MR is a characteristic of a shift from the alternative, anti-

inflammatory M2 to the classical, inflammatory M1 type macrophage. Therefore, a downregulation of MR, associated with an increased abundance of detrimental M1 type macrophages in the AT upon high-fat diet seems plausible. In addition, the finding of MR^- ATMs in mice is particularly interesting since we have used MR as a marker for the isolation of human ATMs. The obvious existence of a MR^- ATM population in mice evokes the question whether there may also be human MR^- ATMs that have not been determined yet. Although this consideration seems interesting, it is rather unprobable since we were never able to detect any MR^- macrophage by immunohistochemistry of human AT sections (118).

In human preadipocyte cultures, we found that OPN-treatment during differentiation led to a decreased adiponectin gene expression in differentiated adipocytes. Since decreased adiponectin expression has been implicated in the pathogenesis of obesity and type 2 diabetes in humans (142), this may indicate that OPN promotes an insulin resistant state by downregulating adiponectin. As however adiponectin expression was not linked to OPN deficiency in mouse experiments (own unpublished observations) and OPN had no other significant effects on insulin resistance and differentiation capacity of adipocytes, we conclude that OPN does not directly influence adipocyte function.

Since we have shown here that OPN is an activator of macrophages and, according to a recent study, OPN is directly mediating ATM accumulation, it may be hypothesized that OPN is affecting insulin resistance via activation and attraction of macrophages. Therefore we investigated if OPN has an effect on adipocyte insulin sensitivity in macrophage - adipocyte coculture systems. Interestingly, OPN-deficient murine ATMs interfere with adipocyte insulin responsiveness as do wt ATMs. This primarily suggests that macrophages do not need OPN to become activated. Another explanation may be that the OPN concentration in the culture medium is sufficient to activate both wild type and OPN-deficient ATMs as a component of an activating cytokine cocktail. Although the main OPN-expressing cell type in human and murine AT is the macrophage, we cannot exclude the possibility that secretion of small amounts of OPN into the medium by 3T3-L1 adipocytes used in our coculture is activating macrophages sufficiently to induce insulin resistance in the adipocyte.
Moreover, the inhibitory effect of macrophages on adipocyte insulin sensitivity was not enhanced by OPN treatment of the coculture. The phenomenon that OPN is an activator

of macrophages but does not induce sufficient activation of the macrophage in order to suppress insulin sensitivity appears surprising. This may partially be explained by the fact that macrophages isolated from the AT, an environment containing multiple cytokines and also OPN, are preactivated and can no longer be stimulated further.

Most probably, however, these results suggest that OPN treatment or deficiency does not have any effect on adipocyte insulin sensitivity in this experimental setup. Although OPN is capable to induce inflammatory reactions in murine and human macrophages, these alterations are, at least *in-vitro*, not directly linked to adipocyte insulin resistance.

In conclusion, improved insulin sensitivity as observed in *spp1* knockout mice is probably not caused by direct action of OPN on adipocytes or activation of ATMs by OPN. In a recent publication (129) it was shown that OPN deficiency impairs ATM accumulation and macrophage migration capacity. As a consequence, it may be considered possible that OPN is acting primarily via macrophage attraction rather than macrophage activation during obesity, but preliminary animal studies of our lab have shown no effect of OPN deficiency on ATM infiltration. Most probably, the mechanism linking OPN to insulin resistance are more complex and may involve additional molecules, cell types or organs. Therefore, other functions of OPN during obesity will have to be further investigated.

6 Conclusion

This thesis identifies OPN as an activator of human macrophages which are strongly supposed to play a role in the development of insulin resistance. OPN leads to increased cytokine production and activation of inflammatory signaling pathways in human model macrophages whereas human ATMs appear to be prestimulated and can only partially be activated further. By contrast, OPN had no profound effect on human or murine adipocytes. Our results indicate that OPN may exert part of its role in obesity-associated adipose tissue inflammation and insulin resistance via induction of the attraction of macrophages into the AT and subsequent inflammatory cytokine production. Since OPN had no effect on insulin sensitivity in macrophage – adipocyte cocultures, the mechanisms that ultimately lead to insulin resistance are presumably more complex and may involve other tissues and organs.

7 Acknowledgements

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I am now to realize that I have come to the end of my thesis. I would like to finish it with a citation that I recalled everytime I lost faith in myself:

„If you think you can do a thing or if you think you can't do a thing, either way you're right.“
Henry Ford, US automobile industrialist (1863 - 1947)

8 References

1. Jequier, E., and Tappy, L. 1999. Regulation of body weight in humans. *Physiol Rev* 79:451-480.
2. Blundell, J.E. 1996. *Regulation of energy intake: appetite control and the potential for weight gain*. London: Libbey. 215-222 pp.
3. Kasper, D.L. 2005. *Harrison's Principles of Internal Medicine*: McGraw-Hill.
4. Schwartz, M.W., Woods, S.C., Seeley, R.J., Barsh, G.S., Baskin, D.G., and Leibel, R.L. 2003. Is the energy homeostasis system inherently biased toward weight gain? *Diabetes* 52:232-238.
5. Neary, N.M., Goldstone, A.P., and Bloom, S.R. 2004. Appetite regulation: from the gut to the hypothalamus. *Clin Endocrinol (Oxf)* 60:153-160.
6. Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., and Flier, J.S. 1996. Role of leptin in the neuroendocrine response to fasting. *Nature* 382:250-252.
7. Kopelman, P.G. 2000. Obesity as a medical problem. *Nature* 404:635-643.
8. Coleman, D.L. 1979. Obesity genes: beneficial effects in heterozygous mice. *Science* 203:663-665.
9. Bjorntorp, P. 2001. Thrifty genes and human obesity. Are we chasing ghosts? *Lancet* 358:1006-1008.
10. Hotamisligil, G.S. 2006. Inflammation and metabolic disorders. *Nature* 444:860-867.
11. Rocchini, A.P. 2002. Childhood obesity and a diabetes epidemic. *N Engl J Med* 346:854-855.
12. 1998. Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults--The Evidence Report. National Institutes of Health. *Obes Res* 6 Suppl 2:51S-209S.
13. Guilbert, J.J. 2003. The world health report 2002 - reducing risks, promoting healthy life. *Educ Health (Abingdon)* 16:230.
14. Bell, C.G., Walley, A.J., and Froguel, P. 2005. The genetics of human obesity. *Nat Rev Genet* 6:221-234.
15. Mokdad, A.H., Marks, J.S., Stroup, D.F., and Gerberding, J.L. 2004. Actual causes of death in the United States, 2000. *Jama* 291:1238-1245.

16. Mokdad, A.H., Ford, E.S., Bowman, B.A., Dietz, W.H., Vinicor, F., Bales, V.S., and Marks, J.S. 2003. Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *Jama* 289:76-79.
17. Gesta, S., Tseng, Y.H., and Kahn, C.R. 2007. Developmental origin of fat: tracking obesity to its source. *Cell* 131:242-256.
18. Kershaw, E.E., and Flier, J.S. 2004. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 89:2548-2556.
19. Ailhaud, G.a.H., H. 2004. Development of white adipose tissue. In *Handbook of Obesity*. G.a.B. Bray, C, editor. New York: Marcel Dekker. 481-514.
20. Rosen, E.D., and Spiegelman, B.M. 2000. Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* 16:145-171.
21. Frayn, K.N., Karpe, F., Fielding, B.A., Macdonald, I.A., and Coppack, S.W. 2003. Integrative physiology of human adipose tissue. *Int J Obes Relat Metab Disord* 27:875-888.
22. Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., and Evans, R.M. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* 83:803-812.
23. Kim, J.B., Sarraf, P., Wright, M., Yao, K.M., Mueller, E., Solanes, G., Lowell, B.B., and Spiegelman, B.M. 1998. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* 101:1-9.
24. Gondret, F., Ferre, P., and Dugail, I. 2001. ADD-1/SREBP-1 is a major determinant of tissue differential lipogenic capacity in mammalian and avian species. *J Lipid Res* 42:106-113.
25. Kissebah, A.H., and Krakower, G.R. 1994. Regional adiposity and morbidity. *Physiol Rev* 74:761-811.
26. Arner, P. 1999. Catecholamine-induced lipolysis in obesity. *Int J Obes Relat Metab Disord* 23 Suppl 1:10-13.
27. Fischer-Posovszky, P., Wabitsch, M., and Hochberg, Z. 2007. Endocrinology of adipose tissue - an update. *Horm Metab Res* 39:314-321.
28. Goralski, K.B., McCarthy, T.C., Hanniman, E.A., Zabel, B.A., Butcher, E.C., Parlee, S.D., Muruganandan, S., and Sinal, C.J. 2007. Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *J Biol Chem* 282:28175-28188.

29. Hutley, L., and Prins, J.B. 2005. Fat as an endocrine organ: relationship to the metabolic syndrome. *Am J Med Sci* 330:280-289.
30. Fantuzzi, G. 2005. Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol* 115:911-919; quiz 920.
31. Klaus, S. 2004. Adipose tissue as a regulator of energy balance. *Curr Drug Targets* 5:241-250.
32. Warne, J.P. 2003. Tumour necrosis factor alpha: a key regulator of adipose tissue mass. *J Endocrinol* 177:351-355.
33. Cianflone, K., Xia, Z., and Chen, L.Y. 2003. Critical review of acylation-stimulating protein physiology in humans and rodents. *Biochim Biophys Acta* 1609:127-143.
34. Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J.S., Tartaglia, L.A., et al. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821-1830.
35. Wang, M.Y., Orci, L., Ravazzola, M., and Unger, R.H. 2005. Fat storage in adipocytes requires inactivation of leptin's paracrine activity: implications for treatment of human obesity. *Proc Natl Acad Sci U S A* 102:18011-18016.
36. Friedman, J.M., and Halaas, J.L. 1998. Leptin and the regulation of body weight in mammals. *Nature* 395:763-770.
37. Havel, P.J. 2004. Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism. *Diabetes* 53 Suppl 1:S143-151.
38. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., et al. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8:1288-1295.
39. Boden, G., Chen, X., Mozzoli, M., and Ryan, I. 1996. Effect of fasting on serum leptin in normal human subjects. *J Clin Endocrinol Metab* 81:3419-3423.
40. Cianflone, K., Lu, H., Smith, J., Yu, W., and Wang, H. 2005. Adiponectin, acylation stimulating protein and complement C3 are altered in obesity in very young children. *Clin Endocrinol (Oxf)* 62:567-572.
41. Roytblat, L., Rachinsky, M., Fisher, A., Greemberg, L., Shapira, Y., Douvdevani, A., and Gelman, S. 2000. Raised interleukin-6 levels in obese patients. *Obes Res* 8:673-675.

42. Hotamisligil, G.S., Arner, P., Caro, J.F., Atkinson, R.L., and Spiegelman, B.M. 1995. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409-2415.

43. Eriksson, P., Reynisdottir, S., Lonnqvist, F., Stemme, V., Hamsten, A., and Arner, P. 1998. Adipose tissue secretion of plasminogen activator inhibitor-1 in non-obese and obese individuals. *Diabetologia* 41:65-71.

44. Bjorbaek, C., and Kahn, B.B. 2004. Leptin signaling in the central nervous system and the periphery. *Recent Prog Horm Res* 59:305-331.

45. Flier, J.S. 2004. Obesity wars: molecular progress confronts an expanding epidemic. *Cell* 116:337-350.

46. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., and Matsubara, K. 1996. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem Biophys Res Commun* 221:286-289.

47. Scherer, P.E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H.F. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 270:26746-26749.

48. Nakano, Y., Tobe, T., Choi-Miura, N.H., Mazda, T., and Tomita, M. 1996. Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J Biochem (Tokyo)* 120:803-812.

49. Hu, E., Liang, P., and Spiegelman, B.M. 1996. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 271:10697-10703.

50. Chandran, M., Phillips, S.A., Ciaraldi, T., and Henry, R.R. 2003. Adiponectin: more than just another fat cell hormone? *Diabetes Care* 26:2442-2450.

51. Diez, J.J., and Iglesias, P. 2003. The role of the novel adipocyte-derived hormone adiponectin in human disease. *Eur J Endocrinol* 148:293-300.

52. Fain, J.N., Madan, A.K., Hiler, M.L., Cheema, P., and Bahouth, S.W. 2004. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 145:2273-2282.

53. Hotamisligil, G.S. 2003. Inflammatory pathways and insulin action. *Int J Obes Relat Metab Disord* 27 Suppl 3:S53-55.

54. Hotamisligil, G.S., Shargill, N.S., and Spiegelman, B.M. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87-91.

55. Ruan, H., and Lodish, H.F. 2003. Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. *Cytokine Growth Factor Rev* 14:447-455.

56. Fernandez-Real, J.M., and Ricart, W. 2003. Insulin resistance and chronic cardiovascular inflammatory syndrome. *Endocr Rev* 24:278-301.

57. Fried, S.K., Bunkin, D.A., and Greenberg, A.S. 1998. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab* 83:847-850.

58. Sartipy, P., and Loskutoff, D.J. 2003. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 100:7265-7270.

59. Takahashi, K., Mizuarai, S., Araki, H., Mashiko, S., Ishihara, A., Kanatani, A., Itadani, H., and Kotani, H. 2003. Adiposity elevates plasma MCP-1 levels leading to the increased CD11b-positive monocytes in mice. *J Biol Chem* 278:46654-46660.

60. Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., and Ferrante, A.W., Jr. 2003. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796-1808.

61. Wellen, K.E., and Hotamisligil, G.S. 2003. Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest* 112:1785-1788.

62. Juge-Aubry, C.E., Somm, E., Pernin, A., Alizadeh, N., Giusti, V., Dayer, J.M., and Meier, C.A. 2005. Adipose tissue is a regulated source of interleukin-10. *Cytokine* 29:270-274.

63. Esposito, K., Pontillo, A., Giugliano, F., Giugliano, G., Marfella, R., Nicoletti, G., and Giugliano, D. 2003. Association of low interleukin-10 levels with the metabolic syndrome in obese women. *J Clin Endocrinol Metab* 88:1055-1058.

64. Pinderski, L.J., Fischbein, M.P., Subbanagounder, G., Fishbein, M.C., Kubo, N., Cheroutre, H., Curtiss, L.K., Berliner, J.A., and Boisvert, W.A. 2002. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. *Circ Res* 90:1064-1071.

65. van Exel, E., Gussekloo, J., de Craen, A.J., Frolich, M., Bootsma-Van Der Wiel, A., and Westendorp, R.G. 2002. Low production capacity of interleukin-10 associates with the metabolic syndrome and type 2 diabetes : the Leiden 85-Plus Study. *Diabetes* 51:1088-1092.

66. Pessin, J.E., and Saltiel, A.R. 2000. Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest* 106:165-169.

67. Saltiel, A.R., and Kahn, C.R. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799-806.

68. Patti, M.E., and Kahn, C.R. 1998. The insulin receptor--a critical link in glucose homeostasis and insulin action. *J Basic Clin Physiol Pharmacol* 9:89-109.

69. White, M.F. 1998. The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem* 182:3-11.

70. Lietzke, S.E., Bose, S., Cronin, T., Klarlund, J., Chawla, A., Czech, M.P., and Lambright, D.G. 2000. Structural basis of 3-phosphoinositide recognition by pleckstrin homology domains. *Mol Cell* 6:385-394.

71. Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., and Cohen, P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* 7:261-269.

72. Cross, D.A., Alessi, D.R., Vandenheede, J.R., McDowell, H.E., Hundal, H.S., and Cohen, P. 1994. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem J* 303 (Pt 1):21-26.

73. Nakae, J., Park, B.C., and Accili, D. 1999. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. *J Biol Chem* 274:15982-15985.

74. Ribon, V., and Saltiel, A.R. 1997. Insulin stimulates tyrosine phosphorylation of the proto-oncogene product of c-Cbl in 3T3-L1 adipocytes. *Biochem J* 324 (Pt 3):839-845.

75. Ribon, V., Herrera, R., Kay, B.K., and Saltiel, A.R. 1998. A role for CAP, a novel, multifunctional Src homology 3 domain-containing protein in formation of actin stress fibers and focal adhesions. *J Biol Chem* 273:4073-4080.

76. Chiang, S.H., Baumann, C.A., Kanzaki, M., Thurmond, D.C., Watson, R.T., Neudauer, C.L., Macara, I.G., Pessin, J.E., and Saltiel, A.R. 2001. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature* 410:944-948.

77. Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., and Yancopoulos, G.D. 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65:663-675.

78. Hotamisligil, G.S., Peraldi, P., Budavari, A., Ellis, R., White, M.F., and Spiegelman, B.M. 1996. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271:665-668.

79. Kahn, S.E., Hull, R.L., and Utzschneider, K.M. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444:840-846.

80. Shoelson, S.E., Lee, J., and Goldfine, A.B. 2006. Inflammation and insulin resistance. *J Clin Invest* 116:1793-1801.

81. Scherer, P.E. 2006. Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes* 55:1537-1545.

82. Wellen, K.E., and Hotamisligil, G.S. 2005. Inflammation, stress, and diabetes. *J Clin Invest* 115:1111-1119.

83. Randle, P.J., Garland, P.B., Hales, C.N., and Newsholme, E.A. 1963. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1:785-789.

84. Shulman, G.I. 2000. Cellular mechanisms of insulin resistance. *J Clin Invest* 106:171-176.

85. Dandona, P., Aljada, A., and Bandyopadhyay, A. 2004. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 25:4-7.

86. Yudkin, J.S., Stehouwer, C.D., Emeis, J.J., and Coppack, S.W. 1999. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* 19:972-978.

87. Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D.R., Miles, J.M., Yudkin, J.S., Klein, S., and Coppack, S.W. 1997. Subcutaneous adipose tissue releases

interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 82:4196-4200.

88. Lundgren, C.H., Brown, S.L., Nordt, T.K., Sobel, B.E., and Fujii, S. 1996. Elaboration of type-1 plasminogen activator inhibitor from adipocytes. A potential pathogenetic link between obesity and cardiovascular disease. *Circulation* 93:106-110.
89. Kern, P.A., Saghizadeh, M., Ong, J.M., Bosch, R.J., Deem, R., and Simsolo, R.B. 1995. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest* 95:2111-2119.
90. Shimomura, I., Funahashi, T., Takahashi, M., Maeda, K., Kotani, K., Nakamura, T., Yamashita, S., Miura, M., Fukuda, Y., Takemura, K., et al. 1996. Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat Med* 2:800-803.
91. Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S., and Lazar, M.A. 2001. The hormone resistin links obesity to diabetes. *Nature* 409:307-312.
92. Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., and Shimomura, I. 2004. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 114:1752-1761.
93. Lin, Y., Berg, A.H., Iyengar, P., Lam, T.K., Giacca, A., Combs, T.P., Rajala, M.W., Du, X., Rollman, B., Li, W., et al. 2005. The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. *J Biol Chem* 280:4617-4626.
94. Ozawa, K., Miyazaki, M., Matsuhisa, M., Takano, K., Nakatani, Y., Hatazaki, M., Tamatani, T., Yamagata, K., Miyagawa, J., Kitao, Y., et al. 2005. The endoplasmic reticulum chaperone improves insulin resistance in type 2 diabetes. *Diabetes* 54:657-663.
95. Nakatani, Y., Kaneto, H., Kawamori, D., Yoshiuchi, K., Hatazaki, M., Matsuoka, T.A., Ozawa, K., Ogawa, S., Hori, M., Yamasaki, Y., et al. 2005. Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. *J Biol Chem* 280:847-851.

96. Ozcan, U., Cao, Q., Yilmaz, E., Lee, A.H., Iwakoshi, N.N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L.H., and Hotamisligil, G.S. 2004. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306:457-461.

97. Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C.Z., Uysal, K.T., Maeda, K., Karin, M., and Hotamisligil, G.S. 2002. A central role for JNK in obesity and insulin resistance. *Nature* 420:333-336.

98. Aguirre, V., Uchida, T., Yenush, L., Davis, R., and White, M.F. 2000. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 275:9047-9054.

99. Cai, D., Yuan, M., Frantz, D.F., Melendez, P.A., Hansen, L., Lee, J., and Shoelson, S.E. 2005. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 11:183-190.

100. Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z.W., Karin, M., and Shoelson, S.E. 2001. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* 293:1673-1677.

101. Janeway, C. 2001. *Immunobiology*: Garland Science.

102. Abbas, A. 2005. *Cellular and Molecular Immunology*: Elsevier Saunders.

103. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.

104. McGreal, E.P., Miller, J.L., and Gordon, S. 2005. Ligand recognition by antigen-presenting cell C-type lectin receptors. *Curr Opin Immunol* 17:18-24.

105. Goerdt, S., and Orfanos, C.E. 1999. Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity* 10:137-142.

106. Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 23:549-555.

107. Herbert, D.R., Holscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., Leeto, M., Kirsch, R., Hall, P., Mossmann, H., et al. 2004. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity* 20:623-635.

108. Stein, M., Keshav, S., Harris, N., and Gordon, S. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 176:287-292.

109. Willment, J.A., Lin, H.H., Reid, D.M., Taylor, P.R., Williams, D.L., Wong, S.Y., Gordon, S., and Brown, G.D. 2003. Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. *J Immunol* 171:4569-4573.

110. Fain, J.N. 2006. Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. *Vitam Horm* 74:443-477.

111. Cinti, S., Mitchell, G., Barbatelli, G., Murano, I., Ceresi, E., Faloia, E., Wang, S., Fortier, M., Greenberg, A.S., and Obin, M.S. 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 46:2347-2355.

112. Permana, P.A., Menge, C., and Reaven, P.D. 2006. Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. *Biochem Biophys Res Commun* 341:507-514.

113. Lumeng, C.N., Deyoung, S.M., and Saltiel, A.R. 2007. Macrophages block insulin action in adipocytes by altering expression of signaling and glucose transport proteins. *Am J Physiol Endocrinol Metab* 292:E166-174.

114. Lacasa, D., Taleb, S., Keophiphath, M., Miranville, A., and Clement, K. 2007. Macrophage-secreted factors impair human adipogenesis: involvement of proinflammatory state in preadipocytes. *Endocrinology* 148:868-877.

115. Zeyda, M., and Stulnig, T.M. 2007. Adipose tissue macrophages. *Immunol Lett* 112:61-67.

116. Lumeng, C.N., Bodzin, J.L., and Saltiel, A.R. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 117:175-184.

117. Curat, C.A., Miranville, A., Sengenes, C., Diehl, M., Tonus, C., Busse, R., and Bouloumie, A. 2004. From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 53:1285-1292.

118. Zeyda, M., Farmer, D., Todoric, J., Aszmann, O., Speiser, M., Gyori, G., Zlabinger, G.J., and Stulnig, T.M. 2007. Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *Int J Obes (Lond)* 31:1420-1428.

119. Denhardt, D.T., Noda, M., O'Regan, A.W., Pavlin, D., and Berman, J.S. 2001. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J Clin Invest* 107:1055-1061.

120. Standal, T., Borset, M., and Sundan, A. 2004. Role of osteopontin in adhesion, migration, cell survival and bone remodeling. *Exp Oncol* 26:179-184.

121. Mazzali, M., Kipari, T., Ophascharoensuk, V., Wesson, J.A., Johnson, R., and Hughes, J. 2002. Osteopontin--a molecule for all seasons. *Qjm* 95:3-13.

122. Rangaswami, H., Bulbule, A., and Kundu, G.C. 2006. Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol* 16:79-87.

123. Xu, G., Nie, H., Li, N., Zheng, W., Zhang, D., Feng, G., Ni, L., Xu, R., Hong, J., and Zhang, J.Z. 2005. Role of osteopontin in amplification and perpetuation of rheumatoid synovitis. *J Clin Invest* 115:1060-1067.

124. Rangaswami, H., Bulbule, A., and Kundu, G.C. 2005. JNK1 differentially regulates osteopontin-induced nuclear factor-inducing kinase/MEKK1-dependent activating protein-1-mediated promatrix metalloproteinase-9 activation. *J Biol Chem* 280:19381-19392.

125. Courter, D.L., Lomas, L., Scatena, M., and Giachelli, C.M. 2005. Src kinase activity is required for integrin alphaVbeta3-mediated activation of nuclear factor-kappaB. *J Biol Chem* 280:12145-12151.

126. Philip, S., and Kundu, G.C. 2003. Osteopontin induces nuclear factor kappa B-mediated promatrix metalloproteinase-2 activation through I kappa B alpha /IKK signaling pathways, and curcumin (diferulolylmethane) down-regulates these pathways. *J Biol Chem* 278:14487-14497.

127. Lin, Y.H., and Yang-Yen, H.F. 2001. The osteopontin-CD44 survival signal involves activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. *J Biol Chem* 276:46024-46030.

128. Konno, S., Hoshi, T., Taira, T., Plunkett, B., and Huang, S.K. 2005. Endotoxin contamination contributes to the in vitro cytokine-inducing activity of osteopontin preparations. *J Interferon Cytokine Res* 25:277-282.

129. Nomiyama, T., Perez-Tilve, D., Ogawa, D., Gizard, F., Zhao, Y., Heywood, E.B., Jones, K.L., Kawamori, R., Cassis, L.A., Tschop, M.H., et al. 2007. Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *J Clin Invest* 117:2877-2888.

130. Denhardt, D.T., Giachelli, C.M., and Rittling, S.R. 2001. Role of osteopontin in cellular signaling and toxicant injury. *Annu Rev Pharmacol Toxicol* 41:723-749.

131. Kiefer, F.W., Zeyda, M., Todoric, J., Huber, J., Geyeregger, R., Weichhart, T., Aszmann, O., Ludvik, B., Silberhumer, G.R., Prager, G., et al. 2008. Osteopontin expression in human and murine obesity: extensive local up-regulation in adipose tissue but minimal systemic alterations. *Endocrinology* 149:1350-1357.

132. Teti, A., Farina, A.R., Villanova, I., Tiberio, A., Tacconelli, A., Sciortino, G., Chambers, A.F., Gulino, A., and Mackay, A.R. 1998. Activation of MMP-2 by human GCT23 giant cell tumour cells induced by osteopontin, bone sialoprotein and GRGDSP peptides is RGD and cell shape change dependent. *Int J Cancer* 77:82-93.

133. Xu, G., Sun, W., He, D., Wang, L., Zheng, W., Nie, H., Ni, L., Zhang, D., Li, N., and Zhang, J. 2005. Overexpression of osteopontin in rheumatoid synovial mononuclear cells is associated with joint inflammation, not with genetic polymorphism. *J Rheumatol* 32:410-416.

134. Isoda, K., Kamezawa, Y., Ayaori, M., Kusuhara, M., Tada, N., and Ohsuzu, F. 2003. Osteopontin transgenic mice fed a high-cholesterol diet develop early fatty-streak lesions. *Circulation* 107:679-681.

135. Matsui, Y., Jia, N., Okamoto, H., Kon, S., Onozuka, H., Akino, M., Liu, L., Morimoto, J., Rittling, S.R., Denhardt, D., et al. 2004. Role of osteopontin in cardiac fibrosis and remodeling in angiotensin II-induced cardiac hypertrophy. *Hypertension* 43:1195-1201.

136. Todoric, J., Loffler, M., Huber, J., Bilban, M., Reimers, M., Kadl, A., Zeyda, M., Waldhausl, W., and Stulnig, T.M. 2006. Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia* 49:2109-2119.

137. Brown, J.M., Boysen, M.S., Jensen, S.S., Morrison, R.F., Storkson, J., Lea-Currie, R., Pariza, M., Mandrup, S., and McIntosh, M.K. 2003. Isomer-specific

regulation of metabolism and PPARgamma signaling by CLA in human preadipocytes. *J Lipid Res* 44:1287-1300.

138. Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R., and Pfeiffer, E.F. 1989. Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J Clin Invest* 84:1663-1670.
139. Barzilai, N., She, L., Liu, L., Wang, J., Hu, M., Vuguin, P., and Rossetti, L. 1999. Decreased visceral adiposity accounts for leptin effect on hepatic but not peripheral insulin action. *Am J Physiol* 277:E291-298.
140. Hotamisligil, G.S., Murray, D.L., Choy, L.N., and Spiegelman, B.M. 1994. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. *Proc Natl Acad Sci U S A* 91:4854-4858.
141. Oyama, Y., Akuzawa, N., Nagai, R., and Kurabayashi, M. 2002. PPARgamma ligand inhibits osteopontin gene expression through interference with binding of nuclear factors to A/T-rich sequence in THP-1 cells. *Circ Res* 90:348-355.
142. Statnick, M.A., Beavers, L.S., Conner, L.J., Corominola, H., Johnson, D., Hammond, C.D., Rafaeloff-Phail, R., Seng, T., Suter, T.M., Sluka, J.P., et al. 2000. Decreased expression of apM1 in omental and subcutaneous adipose tissue of humans with type 2 diabetes. *Int J Exp Diabetes Res* 1:81-88.

9 Used Abbreviations

ADD	adipocyte determination and differentiation factor
AP	activator protein
APS	ammonium persulfate
AT	adipose tissue
ATM	adipose tissue macrophage
BAT	brown adipose tissue
BCA	bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumine
CAP	Cbl-associated protein
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CCL	chemokine ligand
CNS	central nervous system
CRP	C-reactive protein
DG	Deoxyglucose
DMEM	Dulbecco’s modified Eagle’s medium
dNTP	deoxyribonucleotide triphosphate
DTT	dithiotreitole
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FBS	fetal bovine serum
FCS	fetal calf serum
GLUT	glucose transporter
Grb2	growth factor receptor-bound protein 2
HBSS	Hank’s buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high-fat diet
HRP	horseradish peroxidase

IBMX	isobutylmethylxanthine
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IκB	inhibitor of NF-κB
IKK	IκB kinase
IRAK	interleukin-1 receptor associated kinase
IRS	insulin-receptor substrate
JNK	c-Jun N-terminal kinase
KRPH	Krebs Ringer phosphate HEPES buffer
LFD	low-fat diet
LIPE	hormone-sensitive lipase
LPS	lipopolysaccharide
M1	classically activated macrophage
M2	alternatively activated macrophage
MACS	magnetic-activated cell separation
MAP	mitogen-activated protein
Mcp	monocyte chemoattractant protein
MEKK	mitogen ERK kinase kinase
MFI	mean fluorescence intensity
MR	mannose receptor
NEFA	nonesterified fatty acid
NF-κB	nuclear factor-κB
O/N	overnight
OPN	osteopontin
PAGE	polyacrylamid gel electrophoresis
PAI	plasminogen activator inhibitor
PBMC	peripheral blood mononuclear cell
PBS	Dulbecco’s phosphate buffered saline
PCR	polymerase chain reaction
PH	pleckstrin homology
PI(3)K	phosphatidylinositol-3-kinase
PKB	protein kinase B
PPAR	peroxisome proliferator-activated receptor

PTPase	protein tyrosine phosphatase
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-qPCR	quantitative real-time PCR
SDS	sodium dodecyl sulfate
SE	standard error
SH2	Src homology 2
Shc	Src homologous and collagen protein
SLC2A4	solute carrier family 2 member 4
SPP	secreted phosphoprotein
SREBP	sterol regulatory element binding protein
SVC	stromal vascular cell
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLR	toll-like receptor
TNF	tumor necrosis factor
WAT	white adipose tissue
wt	wild-type

10 Curriculum vitae

Personal details

Name	Karina Gollinger
Address	Hardtgasse 13/17, 1190 Vienna, Austria
Mobile phone	+4369912927214
e-mail	karina.gollinger@chello.at
Date of birth	July 11, 1984
Place of birth	Vienna
Family status	Unmarried
Nationality	Austria

Education

Sep 2007 – Jun 2008	*Diploma thesis* at the Medical University Vienna
since Oct 2006	Start with 2nd section of Molecular Biology studies
since Oct 2003	*Studies of Molecular Biology*, University Vienna 1st section passed with distinction
Oct 2002 – Sep 2003	*Studies of Chemistry*, University Vienna
Sep 1994 – Jun 2002	*High School*, Sacré Coeur Pressbaum School leaving examination with distinction

Working experience

Sep 2007 – Jun 2008	*Diploma thesis* Department of Internal Medicine III, Clin. Div. Endocrinology and Metabolism, Medical University Vienna (group Thomas Stulnig)
July – Aug 2007	*internship* Boehringer Ingelheim Austria, Division Research/Lead Discovery (group Renate Schnitzer)

April 2007	*internship* Department of Internal Medicine III, Clin. Div. Endocrinology and Metabolism, Medical University Vienna (group Thomas Stulnig)
Feb 2007	*internship* Max F. Perutz Laboratories, Dpt. of Biochemistry, University Vienna (group Cécile Brocard)
Dec 2006	*internship* Department of Internal Medicine III, Clin. Div. Endocrinology and Metabolism, Medical University Vienna (group Thomas Stulnig)
Aug – Sep 2006	*internship* Department of Pathophysiology, Division of Immunopathology, Medical University Vienna (group Rudolf Valenta)
Sep 2005	*internship* Institute for cancer research, Medical University Vienna (group Andrea Gsur)